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Autophagy-related genes in *Helicobacter pylori* infection

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Abstract

Background—*In vitro* studies have shown that *Helicobacter pylori* (*H. pylori*) infection induces autophagy in gastric epithelial cells. However, prolonged exposure to *H. pylori* reduces autophagy by preventing maturation of the autolysosome. The alterations of the autophagy-related genes in *H. pylori* infection are not yet fully understood.

Materials and Methods—We analyzed autophagy-related gene expression in *H. pylori* infected gastric mucosa compared with uninfected gastric mucosa obtained from 136 Bhutanese volunteers with mild dyspeptic symptoms. We also studied single nucleotide polymorphisms (SNPs) of autophagy-related gene in 283 Bhutanese participants to identify the influence on susceptibility to *H. pylori* infection.

Results—Microarray analysis of 226 autophagy-related genes showed that 16 genes were up-regulated (7%) and 9 were down-regulated (4%). We used quantitative reverse transcriptase-polymerase chain reaction to measured mRNA levels of the down-regulated genes (*ATG16L1*, *ATG5*, *ATG4D* and *ATG9A*) that were core molecules of autophagy. *ATG16L1* and *ATG5* mRNA levels in *H. pylori* positive specimens (n = 86) were significantly less than in *H. pylori* negative specimens (n = 50). *ATG16L1* mRNA levels were inversely related to *H. pylori* density. We also compared SNPs of *ATG16L1* (rs2241880) among 206 *H. pylori*-positive and 77 negative subjects. The odds ratio for the presence of *H. pylori* in the GG genotype was 0.40 (95% CI: 0.18-0.91) relative to the AA/AG genotypes.

Conclusions—Autophagy-related gene expression profiling using high-throughput microarray analysis indicated that down-regulation of core autophagy machinery genes may depress autophagy functions and possibly provide a better intracellular habit for *H. pylori* in gastric epithelial cells.

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Disclosures

Competing interests: The authors have no financial conflicts of interest. Dr. Graham is an unpaid consultant for Novartis in relation to vaccine development for the treatment or prevention of *Helicobacter pylori* infection. Dr. Graham is a paid consultant for RedHill Biopharma regarding novel *H. pylori* therapies and he has received research support for the culture of *H. pylori*. He is a consultant for Otsuka Pharmaceuticals regarding diagnostic breath testing. Dr. Graham has received royalties from Baylor College of Medicine for patents covering materials related to a ^{13}C-urea breath test.

Introduction

Although *Helicobacter pylori* (*H. pylori*) is generally considered an extracellular human pathogen, *H. pylori* also can reside within gastric epithelial cells [1, 2]. It has been proposed that the ability of *H. pylori* to reside within gastric epithelial cells may be in part responsible for the difficulty in eradicating the infection with antimicrobial therapy [3]. The possibility role of intracellular expression of *H. pylori* genes in the development of *H. pylori* associated diseases remains unclear [4–6].

Macroautophagy (hereafter referred to as autophagy) is an intracellular process in which cytoplasmic material is delivered to lysosomes for degradation [7–9]. Bacterial pathogens are among the targets of selective autophagy, termed xenophagy[10]. Xenophagy is an innate immune mechanism. Autophagy can target intracellular bacteria present in either the cytosol or within vacuoles and restrict their growth. In most cases, autophagosomes form around the target bacteria and deliver them to the lysosome for degradation.

The autophagic process is regulated at both the post-translational and transcriptional level [11, 12]. *In vitro* studies have shown that infection of gastric epithelial cells with *H. pylori* can induce autophagy [13–15]. However, prolonged exposure (e.g., for 24 hours) of these cells to culture supernatants from vacuolating cytotoxin A (VacA) positive *H. pylori* results in prevention of autolysosome maturation resulting in an overall reduction in autophagy[16]. Tang et al.[17] showed that the expression of the key autophagy genes *ATG12* and *BECN1* decreased in association with up-regulation of *microRNA (MIR) 30B* in the gastric mucosa. They also found that conversion of LC3B-I to LC3B-II required for autophagy function was reduced in *H. pylori*-infected gastric mucosa compared to uninfected mucosa suggesting that the *MIR30B*-related reduction in autophagy function allowed intracellular *H. pylori* to evade autophagic clearance.

Despite recent interest changes in autophagy-related gene expression during *H. pylori* infection, the effect of *H. pylori* on autophagy function remains poorly understood. We used the Human Autophagy Database (HADb; available at www.autophagy.lu) and microarray analysis to analyze expression of autophagy-related genes in *H. pylori*-infected and uninfected human gastric mucosa. We also studied the relationship of single nucleotide polymorphisms (SNPs) of down-regulated autophagy-related genes in relation to susceptibility to *H. pylori* infection.

Methods

Subjects and gastric biopsy specimens

We used gastric mucosal biopsy specimens obtained from *H. pylori* infected and uninfected volunteers in Bhutan obtained as previously described [18]. In a previous study, we recruited a total of 372 volunteers with mild dyspeptic symptoms from three Bhutanese cities (Thimphu, Punaka, and Wangdue) during four days (December 6 to December 9) in 2010. During endoscopy, 4 gastric biopsy specimens were collected from healthy areas in the antrum: one each for *H. pylori* culture followed by DNA extraction, rapid urease test, RNA

analysis, and histological examination. Written informed consent was obtained from all participants, and the protocol was approved by the ethics committee of Oita University (Japan) and Chulalongkorn University (Thailand) as well by the local hospitals where we collected the specimens[18].

All biopsy specimens for culture and RNA analyses were immediately placed in a −20°C freezer and subsequently sent on dry ice by Express Mail to Oita University Faculty of Medicine, Japan, where they were stored at −80°C until use. Biopsy specimens for histology were fixed in buffered formalin at room temperature and were sent to Oita University Faculty of Medicine for sectioning and analyses. Total RNA from the gastric specimens placed in RNA later (Ambion, Life Technologies, Carlsbad, CA) was isolated using commercially available kits (Ambion) and genomic DNA was isolated from gastric specimens following the *H. pylori* culture using DNeasy Blood & Tissue Kit (Qiagen).

H. pylori culture and status

H. pylori culture was performed using standard culture methods, as previously described[19]. *H. pylori* status was determined using the combination of rapid urease test, serology, culture, and histology. Subjects were considered to be *H. pylori*-negative when all four tests were negative and as *H. pylori*-positive when at least two of these examinations yielded positive results.

cagA and *vacA* genotyping of *H. pylori*

H. pylori DNA was extracted from *H. pylori* cultured on plates using the commercially available kit (DNeasy Blood & Tissue Kit; Qiagen, Valencia, CA). The *cagA* status was determined by polymerase chain reaction (PCR) for a conserved region of *cagA* and for direct sequence[20]. The *cagA* genotype (East-Asian type and Western type) was confirmed by sequencing the PCR products as described previously[21]. The *vacA* genotyping (s1, s2, m1 and m2) was also performed as described previously[22].

Histology

Biopsy specimens for histological examination were fixed in 10% (vol/vol) neutralized buffered formalin, embedded in paraffin wax and stained with hematoxylin-eosin and Giemsa stains. Specimens were evaluated by a histologist blinded to the clinical features of patients or the characteristics of the *H. pylori* strains. *H. pylori* density, the degree of mononuclear cell (MNC, inflammation) and polymorphonuclear leucocyte infiltration (PMN, activity) were determined according to the updated Sydney system. The *H. pylori* density was also evaluated by immunohistochemistry with polyclonal anti-*H. pylori* antibody, as described previously[23]. The *H. pylori* density was scored based on the average density on the surface and the foveolar epithelium. If areas with widely different scores were obtained on the same specimen, an average based on the general evaluation of the biopsy was considered. Only areas without metaplasia were evaluated for the presence of *H. pylori*.

Gene expression microarrays

Gene expression levels from the gastric specimens were analyzed by gene expression microarray. Complementary RNA was amplified, labeled, and hybridized to a 44K Agilent 60-mer oligo microarray according to the manufacturer's instructions. All hybridized microarray slides were scanned using an Agilent scanner, and relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Differences in mRNA expressions between the two groups were considered significant if the fold change of expression values was >1.5 and the p value was <0.01 using the t test. The microarray data were registered in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/info/linking.html); the accession number is GSE47797.

Quantitative reverse transcription PCR

Expression levels of mRNAs that showed significant differences based on the microarray results were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) from gastric biopsies. qRT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Beta-actin (*ACTB*) was used as the endogenous control for data normalization. Predesigned TaqMan Gene Expression Assays including primer set and TaqMan probe (*ATG16L1*: Hs01003142_m1, *ATG5*: Hs00169468_m1, *ATG9A*: Hs01036946_m1, *ATG4D*: Hs00262792_m1, *IL1B*: Hs01555410_m1, *ACTB*: Hs01060665_g1) were purchased from Applied Biosystems. mRNA levels of these genes in gastric specimens were quantified using ABI Prism 7300 sequence detection system (Applied Biosystems). The samples were placed in the analyzer and PCR was conducted according to the manufacturer’s instructions. The ratio change in target gene relative to the endogenous control gene (*ACTB*) was determined by the $2^{-\Delta\Delta CT}$ method.

SNPs genotyping analysis

TaqMan SNP Genotyping Assays (*ATG16L1*: rs2241880) designed with two specific probes and primers for each variant were purchased from Applied Biosystems. Genomic DNA was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) and the ABI Prism 7300 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. Analysis of the results was done using the SDS Software version 1.3 (Applied Biosystems). Genotyping data were acquired by a researcher blinded to all clinical information.

Cell culture and *H. pylori* culture used for *in vitro* studies

The human gastric epithelial cancer cell line AGS was obtained from American Type Culture Collection and human gastric epithelial cancer cell lines MKN45 and MKN28 were obtained from Riken Cell Bank (Tsukuba, Japan). Cells were routinely maintained in RPMI 1640 medium (Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin at 37°C and 5% CO_2.

In an attempt to investigate how *H. pylori* might regulate *ATG16L1* and *ATG5* mRNA levels in the gastric mucosa, we examined the effect of *H. pylori* infection on human gastric epithelial cancer cells. First, we examined three gastric epithelial cancer cell lines (AGS, MKN45 and MKN28) for the expression of *ATG16L1* mRNA using qRT-PCR. In the second step, *H. pylori* strain 26695 was used to infect MKN45 and MKN28 for different time periods (3, 6 and 24h) and at different multiplicity of infections (MOIs) of 50 and 100. *H. pylori* strain 26695 was used from our stocks. *H. pylori* were cultured on Brain Heart Infusion (BHI; Becton, Dickinson, and Company, Sparks, MD) agar plates containing 7% defibrinated horse blood for 72 h. Before infection, bacteria were inoculated into BHI broth with 10% FBS and grown under microaerophilic conditions at 37°C overnight with shaking. Bacteria were washed with phosphate-buffered saline (PBS) (pH 7.4), resuspended in PBS for the duration of infection, and used to infect cell cultures. Cultured cells were infected with *H. pylori* at a multiplicity of infection (MOI) of 50 or 100. Total RNA from human cell lines was extracted using commercially available kits (Ambion).

Western blotting

Cells were washed with ice-cold PBS and then solubilized in a buffer containing 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EGTA, protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany) and cleared by centrifugation at 10000 g. Total protein was separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk (Bio-Rad) for 1 h and then incubated with primary antibodies. The primary antibodies used were rabbit anti-LC3B (L7543, 1:1000, Sigma-Aldrich), and goat anti-β-actin (sc-1616, 1:500; Santa Cruz Biotechnology). After washing, the membranes were incubated in HRP-conjugated secondary antibodies. The protein signals were detected using the Clarity Western ECL Substrate (Bio-Rad).

Statistics

All statistical analyses were performed by JMP 10.0 software (SAS, Cary, NC) or Microsoft Excel for Mac 2011 (version 14.4.8, Microsoft). Clinical samples were analyzed using the χ^2 test to compare discrete variables and the Mann-Whitney U test to compare continuous variables. *In vitro* samples were analyzed using Student's *t*-test. Correlation coefficients were calculated by Spearman rank correlation coefficient. For SNPs analysis, deviations of genotype frequencies from those expected under the Hardy-Weinberg equilibrium (HWE) were assessed by a goodness-of-fit χ^2-test. Logistic regression adjusted by age and sex was used to analyze odds ratio (OR) and 95% confidence interval (CI). All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Subjects

Samples from 136 subjects from Bhutan (median age, 40 years; age range, 16–92 years) were included in this study (Table 1). For gene expression analysis, all samples collected from participants in the capital city Thimphu on December 6th (n = 136) were investigated. For the SNP analysis, 283 of the available samples (n = 372) were investigated, the

remaining samples being excluded due to lack of sufficient sample or background data. These subjects were previously reported in a survey of the prevalence of *H. pylori* infection[18] and the effect of *H. pylori* infection on gastric *interleukin (IL)-8* and *IL-10* mRNA levels[24]. The prevalence of *H. pylori* was 63% and all strains possessed *cagA* (Table 1).

Autophagy related gene expression profiles in *H. pylori* infection

We selected 8 samples for microarray analysis based on typical pathological findings (four samples were obtained from 50 subjects with *H. pylori* negative normal mucosa, and 4 from the 86 subjects with *H. pylori* positive gastritis mucosa). In this study, an array chipset that contained 50,599 total probe sets was used. For comprehensive expression analyses of autophagy-related genes, 226/237 genes in Human Autophagy Data base (HADb) were chosen from the microarray; 11 genes were excluded because 3 genes had no probe in this array chipset and 8 genes were not detected in all samples measured (Figure 1). Determination of the expression levels of the autophagy related genes showed 16/226 (7%) to be up-regulated (*NLRC4*: Fold change [FC] 11.88, $p < 0.001$; *CXCR4*: FC 7.40, $p < 0.001$; *CCL2*: FC 6.22, $p < 0.001$; *GRID1*: FC 3.49, $p = 0.006$; *CX3CL1*: FC 3.29, $p = 0.006$; *BCL2*: FC 2.79, $p = 0.002$; *RGS19*: FC 2.72, $p < 0.001$; *PRKCQ*: FC 2.71, $p = 0.003$; *FAS*: FC 2.55, $p = 0.007$; *ATG16L2*: FC 2.27, $p < 0.001$; *ARSB*: FC 2.26, $p = 0.006$; *CASP1*: FC 2.09, $p = 0.001$; *ITPR1*: FC 2.00, $p = 0.003$; *BID*: FC 1.90, $p = 0.002$; *DNAJB9*: FC 1.88, $p = 0.009$; *RAB24*: FC 1.64, $p = 0.001$) and 9/226 (4%) to be down-regulated (*ATG9A*: FC −1.58, $p = 0.003$; *ITGB4*: FC −1.60, $p = 0.001$; *ATG5*: FC −1.71, $p = 0.005$; *PTK6*: FC −1.72, $p = 0.009$; *ATG16L1*: FC −1.73, $p = 0.004$; *MAPK3*: FC −1.80, $p = 0.002$; *FKBP1B*: FC −2.01, $p < 0.001$; *ATG4D*: FC 251 −2.18, $p < 0.001$; *STBD1*: FC −2.42, $p = 0.006$; respectively). Up-regulation was defined as at least a 1.5 fold increase and down regulation by at least a 1.5-fold decrease. In down-regulated genes, the core components of the autophagy machinery were included (*ATG4D*, *ATG16L1*, *ATG5* and *ATG9A*).

Autophagy-related genes mRNA expression levels in the antral gastric mucosa

We measured the mRNA levels using qRT-PCR of the four down-regulated genes that were core molecules of autophagy: *ATG16L1*, *ATG5*, *ATG4D* and *ATG9A*. *ATG16L1* mRNA levels in *H. pylori* positive specimens (n = 86) were significantly reduced compared to those in *H. pylori* negative specimens (n = 50) (*H. pylori* negative: median 2.34, range 0.281–9.41 and *H. pylori* positive: median 1.65, range 0.105–4.17, respectively; $p < 0.001$) (Figure 2A). *ATG5* mRNA levels in *H. pylori* positive samples were also significantly lower than those in *H. pylori* negative samples (*H. pylori* negative: median 1.96, range 0.110–6.49 and *H. pylori* positive: median 1.32, range 0.171–6.42, respectively; $p = 0.048$) (Figure 2B). *ATG9A* mRNA levels in *H. pylori* positive samples were not statistically reduced compared to those in negative samples (*H. pylori* negative: median 1.04, range 0.068–5.01 and *H. pylori* positive: median 0.693, range 0.079–4.84, respectively; $p = 0.061$) (Figure 2C). *ATG4D* mRNA levels were not significantly different *H. pylori* positive and negative cases (*H. pylori* negative: median 0.553, range 0.050–6.26, and *H. pylori* positive: median 0.807, range 0.021–6.95, respectively; $p = 0.379$) (Figure 2D).

ATG16L1 and *ATG5* mRNA levels and *H. pylori* density

We examined the association between *ATG16L1* and *ATG5* mRNA levels and *H. pylori* density scores as determined using the updated Sydney System. There was a significant inverse relation between *ATG16L1* mRNA levels and *H. pylori* density (score 0 vs 2, $p = 0.008$; score 0 vs 3, $p = 0.002$; score 1 vs 3, $p = 0.046$; respectively) (Figure 3A). On the other hand, there was no significant difference in *ATG5* mRNA levels in relation to the *H. pylori* density score (Figure 3B).

ATG16L1 and *ATG5* mRNA levels and other histological findings

We examined for a possible correlation between *ATG16L1* and *ATG5* mRNA levels and the other histological findings (Table 2) however none of the other histological findings (PMN, MNC, atrophy) were significantly correlated with *ATG16L1* or *ATG5* mRNA levels (*ATG16L1*: PMN, $p = 0.681$; MNC, $p = 0.291$; atrophy, $p = 0.406$; *ATG5*: PMN, $p = 0.155$; MNC, $p = 0.160$; atrophy, $p = 0.142$; respectively).

ATG16L1 and *ATG5* mRNA levels in relation to *vacA* and *ATG16L1* genotype

Among 86 *H. pylori*-infected subjects with our criteria, we were able to culture 76 *H. pylori* strains. We performed *vacA* genotyping on 76 *H. pylori* strains. All strains were s1 genotype (s1m1, n = 54 or s1m2, n = 22) (Table 1). There was no significant difference in *ATG16L1* and *ATG5* mRNA levels between *vacA* s1m1 and s1m2 genotypes (*ATG16L1*, $p = 0.855$; *ATG5*, $p = 0.797$) (Supplementary Figure 1). We genotyped *ATG16L1* SNPs (rs2241880) using TaqMan SNP genotyping assays and analyzed whether there was an association between *ATG16L1* mRNA levels and *ATG16L1* genotype (Supplementary Figure 2). Twelve samples were excluded from the gene expression analysis as the genotype could not be identified (n = 124). No association was found between *ATG16L1* mRNA levels and *ATG16L1* genotype whether using all subjects (n = 124, $p = 0.569$) or specifically *H. pylori* positive subjects (n = 81, $p = 0.930$).

ATG16L1 mRNA levels and the pro-inflammatory cytokine interleukin 1β (IL-1β)

Lee et al.[25] using mouse embryonic fibroblasts reported that ATG16L1 suppressed IL-1β signaling via regulation of p62 stability and mediated ubiquitination of p62. Plantinga et al. [26] used human peripheral blood mononuclear cells to demonstrated that genetic variation in *ATG16L1* was associated with higher production of IL-1β. We examined whether there was an association between *ATG16L1* mRNA levels, genotype and *IL-1β* mRNA levels in *H. pylori* infected and uninfected gastric mucosa and found no correlation between *ATG16L1* mRNA levels and *IL-1β* mRNA levels in either all subjects (n = 124; correlation coefficient, 0.092; $p = 0.286$) or *H. pylori* positive subjects (n = 81; correlation coefficient, 0.102, $p = 0.350$) and no association between *ATG16L1* genotype and *IL-1β* mRNA levels in either all subjects (n = 124, $p = 0.750$) or *H. pylori* positive gastric mucosa (n = 81, $p = 0.828$) (Supplementary Figure 3).

ATG16L1 and *ATG5* mRNA levels in gastric epithelial cells in response to *H. pylori* infection

LC3B-II protein expression (Autophagy function) was increased by *H. pylori* infection *in vitro* (Supplementary Figure 4). Gastric mucosal *ATG16L1* and *ATG5* mRNA levels were reduced in *H. pylori*-infected gastric mucosa compared to uninfected mucosa (Figure 2). These results matched our hypothesis. Given this we investigated how *H. pylori* might regulate *ATG16L1* and *ATG5* mRNA levels in gastric epithelial cells. In a preliminary experiment, *ATG16L1* mRNA was detected in MKN45 and MKN28, but not in AGS cells (data not shown). Based on our *in vivo* data showing an inverse relation between *H. pylori* density and *ATG16L1* expression, we examined the effects on autophagy-related genes during both the early and late phases of infection and at lower and higher MOIs (Figure 4). However, we were unable to demonstrate either a time or MOI-dependency in gene expression with either cell line; MKN45 (Figure 4A, 4B) or MKN28 (Figure 4C, 4D).

The influence on *H. pylori* susceptibility in relation of *ATG16L1* polymorphisms

To evaluate whether there was an association between *ATG16L1* polymorphisms and susceptibility to *H. pylori* infection, we performed a case-control study including 77 *H. pylori* negative and 206 *H. pylori* positive subjects (Supplementary Table 1). The genotype distribution for *ATG16L1*: rs2241880 polymorphism in this study were similar to those expected for Hardy-Weinberg Equilibrium ($p = 0.172$). The risk of being *H. pylori* infected decreased in recessive model (AA+AG vs. GG; OR, 0.40; 95% CI, 0.18-0.91; $p = 0.029$) (Table 3). The AG genotype was marginally associated with higher risk of *H. pylori* infection when compared to AA genotype (OR, 1.83; 95% CI, 1.00-3.42; $p = 0.048$).

Discussion

To the best of our knowledge, this is the first report of detailed expression analyses of autophagy-related genes using microarray analysis to compare *H. pylori*-infected and uninfected human gastric mucosa. It has previously been shown that intracellular bacterial pathogens are capable of suppressing autophagy by down-regulating autophagy-related genes[27]. The present study showed that the core component autophagy-related genes, *ATG16L1* and *ATG5*, mRNA levels were significantly reduced in *H. pylori* positive human gastric mucosa (Figure 2). *ATG16L1* and *ATG5* encode key autophagy proteins which function as part of a complex with ATG12 responsible for the proper subcellular localization of the autophagy machinery[28]. Nguyen et al.[29] used both *in vitro* and a mouse model to described a reduction in ATG16L1 and ATG5 mRNA and protein levels associated with the up-regulation of *MIR30C* and *MIR130A* in intestinal epithelial cells infected with adherent invasive *Escherichia coli*. They also reported that *ATG16L1* and *ATG5* mRNA levels were reduced in ileal biopsy specimens of Crohn's disease patients compared to healthy controls. In our study, we found down-regulation of *ATG16L1* and *ATG5* expression in *H. pylori* infected gastric mucosa which is consistent with the notion that suppression of autophagy could promote *H. pylori* residence within gastric epithelial cells.

We also found that *ATG16L1* mRNA levels in the gastric mucosa decreased in a *H. pylori* density-dependent manner (Figure 3), however we found no correlation between *ATG16L1*

mRNA levels and mucosal infiltration with acute or mononuclear inflammatory cells (Table 2). These results are similar to those of Glas et al. [30] who reported no association between *ATG16L1* mRNA levels between inflamed lesions vs. non-inflamed Crohn's disease tissue biopsies. Together these results suggest that neither the presence, nor the intensity of the acute or chronic inflammatory cell infiltration of the gut mucosa significantly influence *ATG16L1* mRNA levels. However, several studies have suggested that Atg16L1 may be involved in the regulation of inflammatory responses[25, 26]. For example, Saito et al. [31] reported that lipopolysaccharide stimulation of *ATG16L1*-deficient cells resulted in the production of large amounts of proinflammatory cytokines IL-1β and IL-18. However, we found no association between between *ATG16L1* mRNA levels, genotype and *IL-1β* mRNA levels in *H. pylori* infected gastric tissue (Supplementary Figure 3). More studies are needed to examine the precise genetic and functional roles of autophagy-related genes in the pathogenesis of *H. pylori*–induced gastritis.

Based on the microarray analysis, *ATG4D* expression was found to be significantly down-regulated however, using qRT-PCR analyses up-regulation was observed. In previous studies, we have confirmed our microarray data using qRT-PCR with β-actin as the housekeeping gene. In general, our results have been similar [24, 32]. The reason why the qRT-PCR results in the current study do not match that of the microarray data is unclear. However, it is possible that this may relate to the fact we used only one house keeping gene (β-actin). Given that the use of 3−5 reference genes is currently recommended, potentially several reference genes may be required for the qRT-PCR of *ATG4D*. Growing evidence from *in vitro* studies has suggested that *H. pylori* virulence factors, especially VacA, are involved in the host's autophagy response to *H. pylori* infection[13, 14, 16]. We therefore investigated the association between *vacA* genotype and *ATG16L1* and *ATG5* mRNA expression in human gastric mucosa. We found no relation between *ATG16L1* and *ATG5* mRNA levels and infection with either the *vacA* s1m1 or s1m2 genotype (there were no s2m2 genotypes) (Supplementary Figure 1).

Our *in vitro* studies using *H. pylori* infection of human gastric cancer cells also found no relationship between the MOI or phase of infection (early vs. late) and *ATG16L1* and *ATG5* mRNA expression. Deen et al.[3] in their review of the interaction between *H. pylori* infection and host cell autophagic processes based primarily on *in vitro* experiments concluded that different host cell lines and bacterial strains produced different results. In contrast to *in vitro* experiments, *H. pylori* are infrequently seen within gastric mucosal cells *in vivo* suggesting that while invasion of epithelial cells occurs and may be a survival strategy, its role, if any, in the pathogenesis of *H. pylori*-related disease will be difficult to unravel. Our *in vitro* studies do not support a major role. In this study, we used one strain; standard *H. pylori* strain 26695 whose whole genome sequences had been first confirmed. Further studies using other strains will be needed to confirm our results. Autophagy is often dysregulated in a wide spectrum of human cancers[33] and most *in vitro* studies have used cancer cells. Possible studies using normal polarized gastric cells such as gastroids will provide a better model the *in vivo* interactions.

Genome-wide association studies (GWAS) have identified *ATG16L1* SNP (rs2241880), encoding a Thr300Ala amino acid substitution (T300A), as a risk variant for Crohn's

disease[34, 35]. Further studies also suggested that the *ATG16L1* SNP was also associated with susceptibility to *H. pylori* infection[16, 36]. Therefore, we performed two analyses in relation to the *ATG16L1* SNP. First, we analyzed whether there was an association between *ATG16L1* SNP and *ATG16L1* mRNA expression levels. We found no significant differences (Supplementary Figure 2). This result is similar to those of Hampe et al.[34] who demonstrated a lack of an association between ATG16L1 protein expression and *ATG16L1* SNP genotype in Crohn's disease colonic mucosal biopsy specimens. Finally, we conducted a case-control study to investigate the influence of *ATG16L1* SNP on susceptibility to *H. pylori* infection (Table 3) which suggested that the prevalence of *H. pylori* infection was significantly reduced in patients bearing *ATG16L1* rs2241880 GG genotype compared to those bearing the AA/AG genotypes. Interestingly, our result is contrary to a report by Raju et al.[16] who found an increased risk of *H. pylori* infection in Scottish and German subjects bearing the GG genotype. A study in a Chinese population showed that *ATG16L1* rs2241880 mutant homozygote (GG) was not found to increase the risk of gastric cancer while the heterozygote (AG) statistically increased the risk of gastric cancer. Further logistic regression analyses by these authors showed that the G allele significantly increases the risk of gastric cancer (OR: 2.38, 95% CI: 1.34-4.24) and the risk of *H. pylori* infection in these ethnic Chinese individuals (OR, 1.49; 95%CI, 1.02-2.16; $p = 0.041$). These types of association studies are easily confounded by racial or regional differences. For example, the association between *ATG16L1* rs2241880 SNP and susceptibility in Crohn's disease was based on patients from Western countries[34, 35] and was not confirmed by studies in Japanese, South Korean, and Han Chinese patients[37]. Epidemiological findings from Asian countries[38] and mostly European-American countries[39] have suggested that the prevalence of *H. pylori* infection in inflammatory bowel disease (IBD) patients was significantly lower than in non-IBD patients. This finding is consistent with our intriguing finding that the GG genotype at rs2241880 suggested a lower risk of *H. pylori* infection and higher risk of Crohn's disease.

In conclusion, examination of autophagy-related gene expression profiling in human gastric mucosa with *H. pylori* infection showed down-regulation of core autophagy machinery genes may depress autophagy functions and possibly allow *H. pylori* to better reside inside gastric epithelial cells. The presence of rs2241880 GG genotype of *ATG16L1* was associated with a reduced risk of *H. pylori* infection. New *in vitro* models are expected to reveal autophagy mechanism in gastric epithelial cells with *H. pylori* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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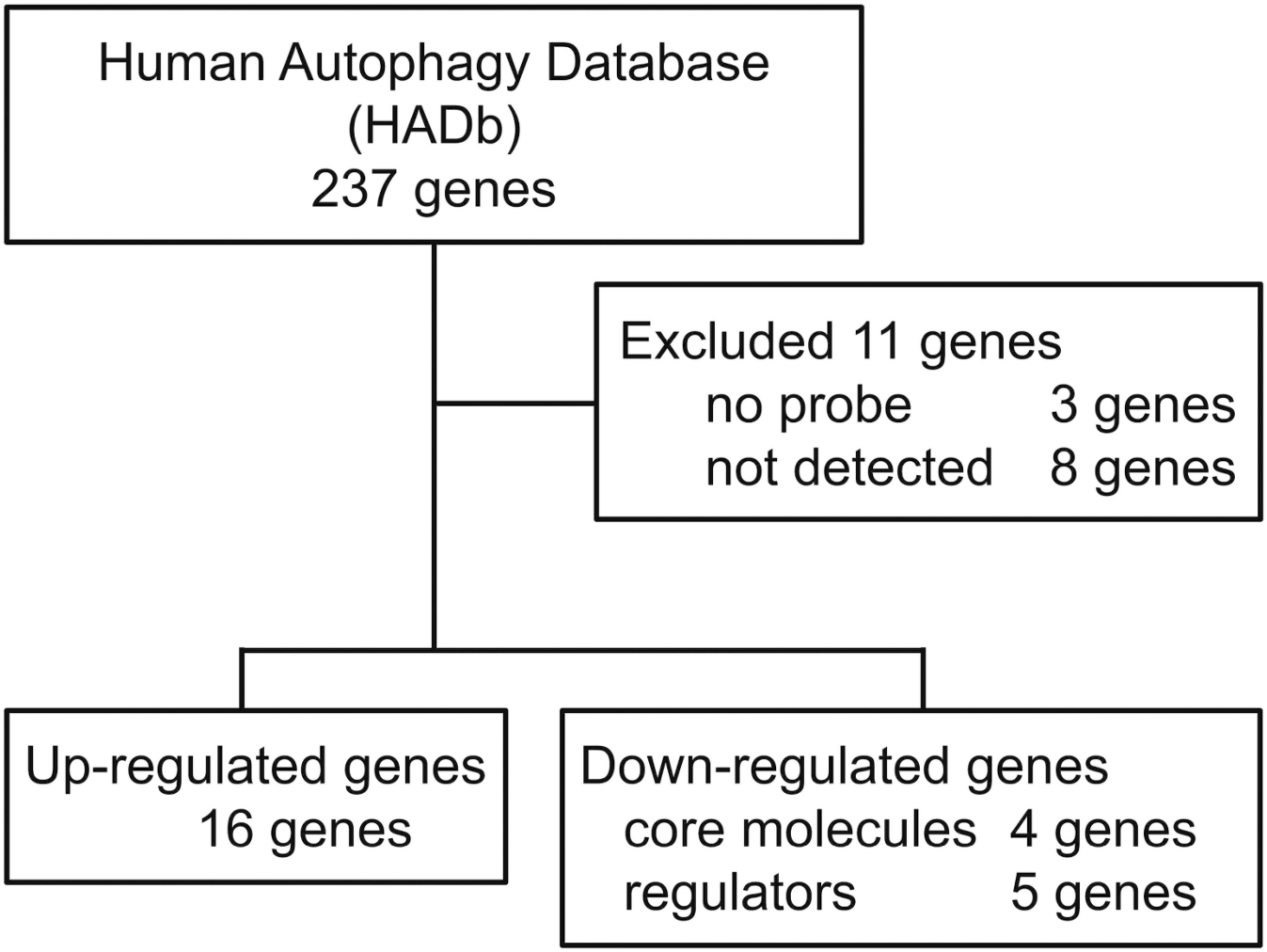

Figure 1.

Flowchart of microarray analysis. Two hundred twenty-six autophagy-related genes in Human Autophagy Database (HADb; http://autophagy.lu/) were analyzed using microarray data (compared 4 *H. pylori* positive and 4 *H. pylori* negative). Differences in autophagy-related genes expression were considered significant if the fold change of expression levels was >1.5 and the *p* value was <0.01.

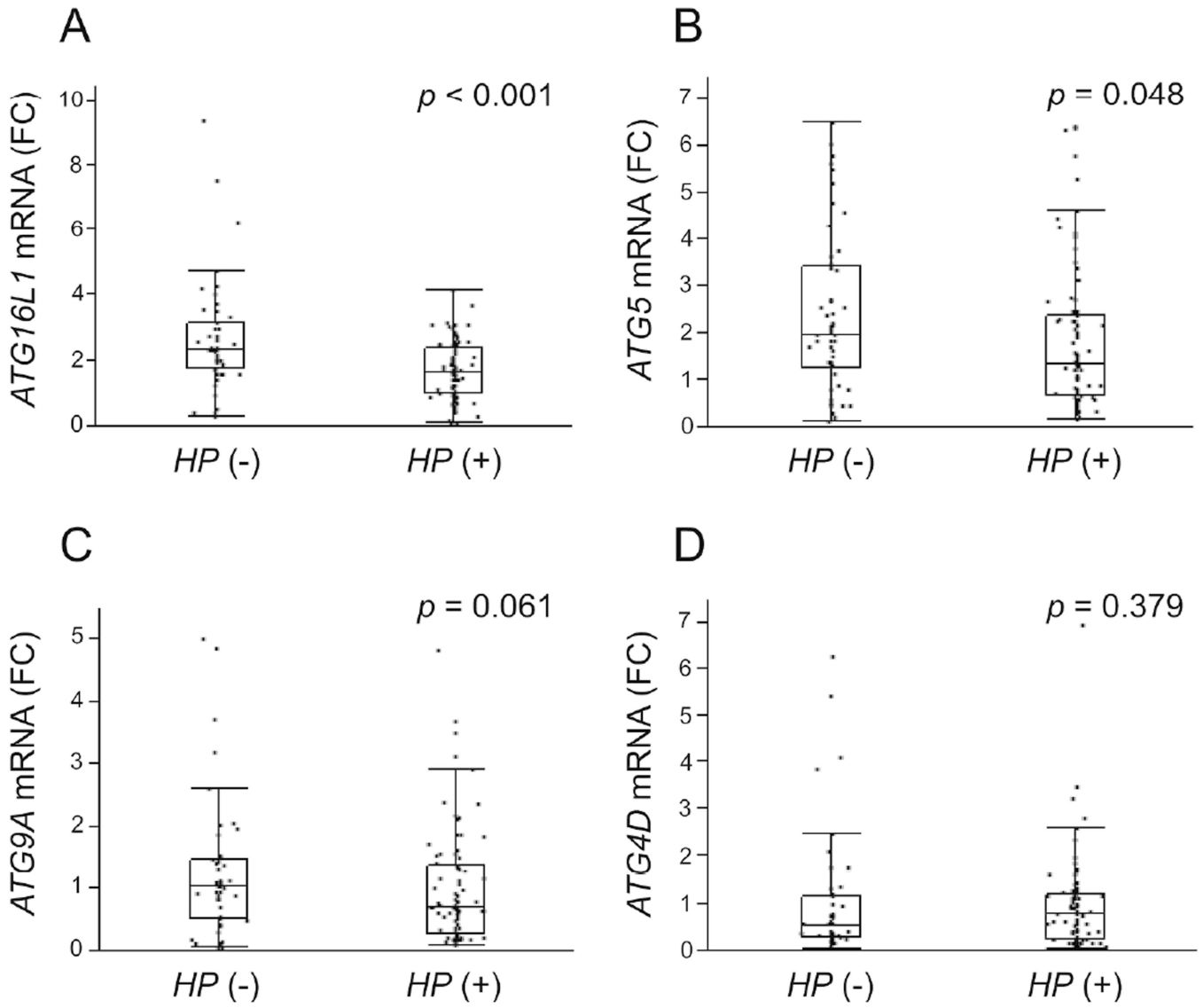

Figure 2.

Autophagy-related genes mRNA levels in the gastric specimens (50 *H. pylori* negative and 86 *H. pylori* positive). Down-regulated core autophagy-related genes (A, *ATG16L1*; B, *ATG5*; C, *ATG9A*; D, *ATG4D*) derived from microarray data were validated by qRT-PCR. Down-regulation of *ATG16L1* and *ATG5* mRNA levels were confirmed respectively. Beta-actin (*ACTB*) was used as the endogenous control for data normalization. Data were expressed by box plotting.

HP,*Helicobacter pylori*; FC, fold change.

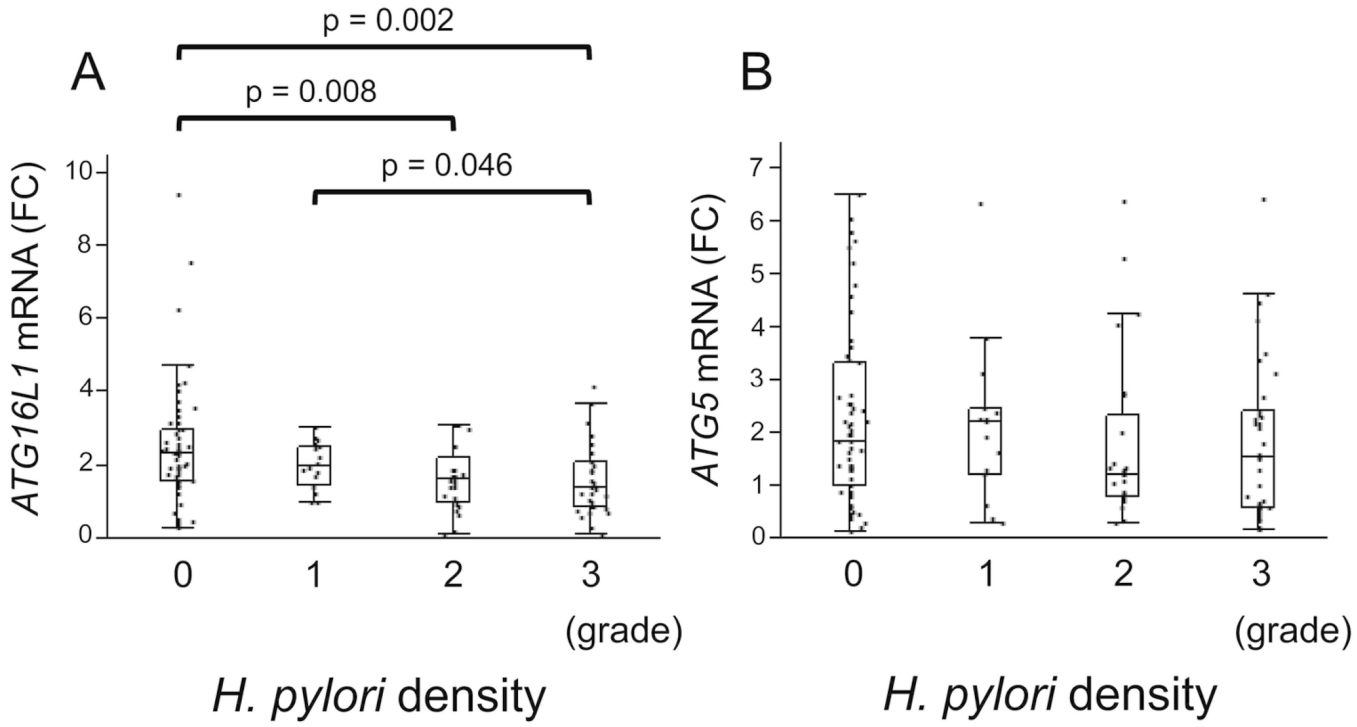

Figure 3.

The association between *ATG16L1* and *ATG5* mRNA levels and *H. pylori* density. The *ATG16L1* mRNA levels were decreased in a step-like manner by *H. pylori* density (Score 0, median 2.33, range 0.281–9.41; Score 1, median 1.99, range 0.997–3.03; score 2, median 1.63, range 0.135–3.12; score 3, median 1.39, range 0.105–4.17). There was no significant difference in *ATG5* mRNA levels in relation to the *H. pylori* density score. *H. pylori* density were scored using the updated Sydney System. *Beta-actin* (*ACTB*) was used as the endogenous control for data normalization. Data were expressed by box plotting. FC, fold change.

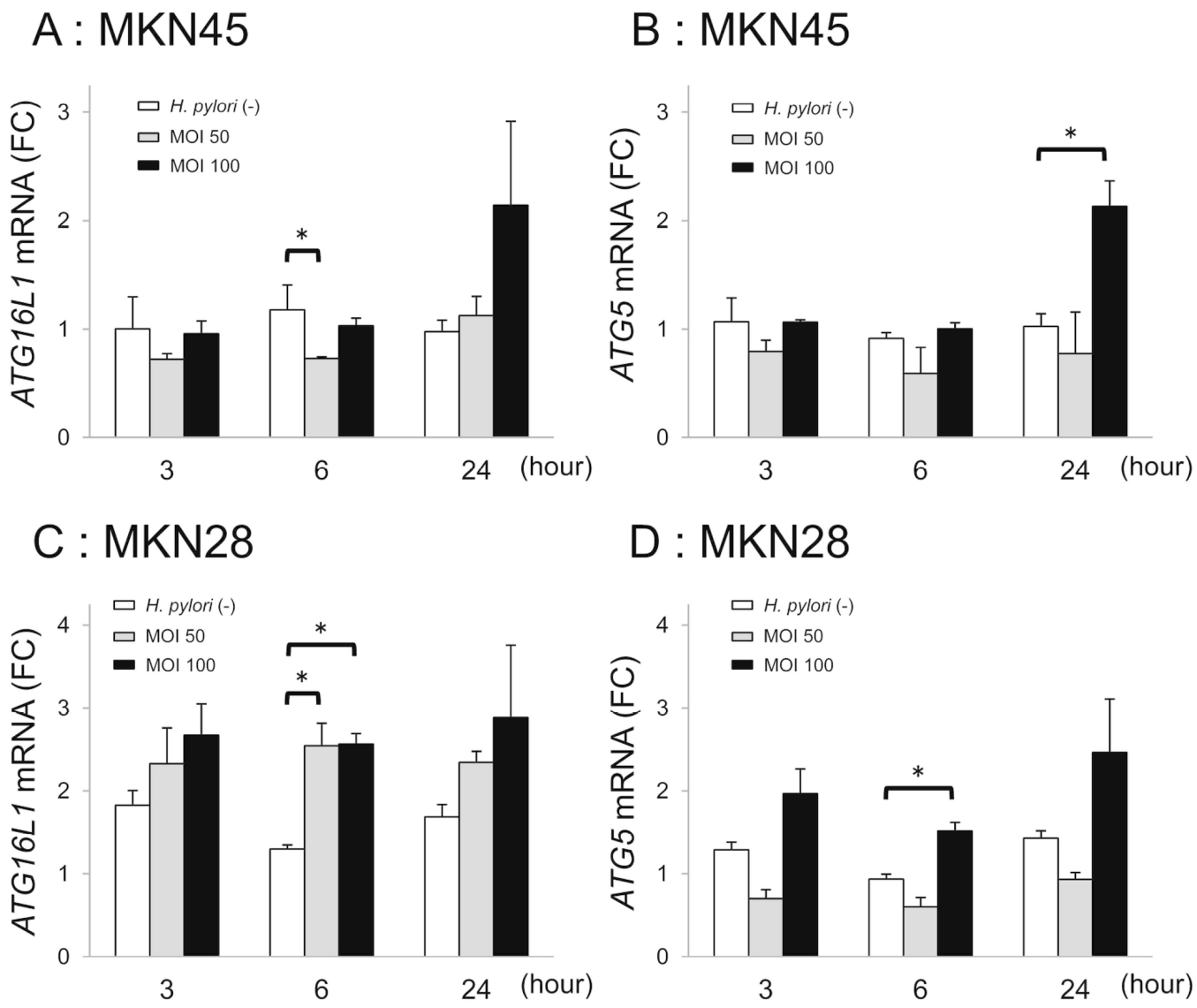

Figure 4.

ATG16L1 and *ATG5* mRNA levels in gastric epithelial cells in response to *H. pylori* infection. A, *ATG16L1* mRNA levels in MKN45 cells. B, *ATG5* mRNA levels in MKN45 cells. C, *ATG16L1* mRNA levels in MKN28 cells. D, *ATG5* mRNA levels in MKN28 cells. Cells were infected with *H. pylori* strain 26695. *ATG16L1* and *ATG5* mRNA levels in *H. pylori*-infected and non-infected MKN45 and MKN28 cells were measured at MOI 50 and 100 for 3, 6 and 24 h. Error bars represent the standard deviation of values obtained from three experiments. Statistical differences between treated and control samples were analyzed using Student's t-test.

* $p < 0.05$.

Table 1

Characteristics of the study subjects and *H. pylori* genotype

	***H. pylori* (−) n=50**	***H. pylori* (+) n=86**	***p* value**
Age, year (range)	45 (18–78)	34 (16–92)	0.003
Male (%)	20 (40)	40 (47)	0.46
cagA			
negative (%)		0 (0)	
Western-type (%)		2 (3)	
East Asian-type (%)		74 (97)	
vacA			
s1m1 (%)		54 (71)	
s1m2 (%)		22 (29)	
s2m1 (%)		0 (0)	
s2m2 (%)		0 (0)	
PMN, Grade			
0 (%)	41 (82)	0 (0)	
1 (%)	8 (16)	42 (49)	
2 (%)	1 (2)	37 (43)	
3 (%)	0 (0)	7 (8)	
Mean, Median	0.200, 0	1.593, 2	<0.001
MNC, Grade			
0 (%)	16 (32)	0 (0)	
1 (%)	33 (66)	20 (23)	
2 (%)	1 (2)	55 (64)	
3 (%)	0 (0)	11 (13)	
Mean, Median	0.700, 1	1.895, 2	<0.001
Atrophy, Grade			
0 (%)	6 (12)	1 (1)	
1 (%)	35 (70)	46 (53)	
2 (%)	6 (12)	35 (41)	
3 (%)	3 (6)	4 (5)	
Mean, Median	1.120, 1	1.488, 1	<0.001
H. pylori density			
0 (%)	50 (100)	9 (10)	
1 (%)	0 (0)	16 (19)	
2 (%)	0 (0)	25 (29)	
3 (%)	0 (0)	36 (42)	
Mean, Median	0, 0	2.023, 2	<0.001

PMN, polymorphonuclear leucocyte; MNC, mononuclear cell.

Table 2

Correlation between *ATG16L1* and *ATG5* mRNA levels and histological findings

ATG16L1			***ATG5***		
	Correl. Coef.	***p* value**		**Correl. Coef.**	***p* value**
PMN	0.045	0.681	PMN	−0.156	0.155
MNC	−0.115	0.291	MNC	−0.154	0.160
atrophy	−0.091	0.406	atrophy	−0.161	0.142

Correl.Coef., Correlation coefficient; PMN, polymorphonuclear leucocyte; MNC, mononuclear cell.

Table 3

The influence on *H. pylori* susceptibility in relation of *ATG16L1* SNP (rs2241880)

Genotype	*H. pylori* negative	*H. pylori* positive	OR (95%CI)[a]	*p* value
***ATG16L1*, rs2241880**	**(%), n=77**	**(%), n=206**		
AA	43 (56)	99 (48)	1.00 (Ref)	
AG	21 (27)	89 (43)	1.83 (1.00–3.42)	0.048
GG	13 (17)	18 (9)	0.53 (0.23–1.22)	0.134
AA	43 (56)	99 (48)	1.00 (Ref)	
AG/GG	34 (44)	107 (52)	1.32 (0.77–2.28)	0.311
AA/AG	64 (83)	188 (91)	1.00 (Ref)	
GG	13 (17)	18 (9)	0.40 (0.18–0.91)	0.029
A allele	107 (69)	287 (70)	1.00 (Ref)	
G allele	47 (31)	125 (30)	0.99 (0.66–1.48)	0.967

OR, odds ratio; CI, confidence interval; Ref, reference.

[a]Adjusted for age and sex in logistic regression model.