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ORIGINAL ARTICLE

Helicobacter pylori dysregulates the expression of autophagy-related genes in human gastric adenocarcinoma cell line in vitro

Marzieh Esmaeilzadeh[1], Farshid Kafilzadeh[1], Mohammad Kargar[2], Hamid Asadzadeh Aghdaei[3], Abbas Yadegar[4*]

[1]Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran
[2]Department of Microbiology, Zand Institute of Higher Education, Shiraz, Iran
[3]Basic and Molecular Epidemiology of Gastrointestinal Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background and Objectives: Studies have indicated that *Helicobacter pylori* (*H. pylori*) infection could correlate with autophagy dysregulation. This research was undertaken to investigate whether *H. pylori* can dysregulate the expression of genes related to autophagy in human gastric adenocarcinoma (AGS) cells.

Materials and Methods: Ten *H. pylori* clinical isolates recovered from peptic ulcer disease (PUD) and chronic gastritis (CG) patients were used for cell infection assays. AGS cells infected with *H. pylori* strains at a multiplicity of infection (MOI) of 100 were incubated at 37°C for 12 h. The expression of autophagy-related genes (*atg5, atg12, atg16L1, LC3B,* and *beclin-1*) was determined in AGS cells by RT-qPCR. ELISA was applied to measure IL-8 production.

Results: The gene expression of *atg5, atg12, atg16L1, LC3B* was upregulated by both CG and PUD strains. The overexpression was more pronounced in PUD than CG strains. On the contrary, *beclin-1* gene was downregulated in all *H. pylori*-infected AGS cells. In addition, *H. pylori* strains could significantly produce IL-8 in AGS cells.

Conclusion: Our in vitro study demonstrates that *H. pylori* could alter the expression of autophagy-related genes. Further investigation could precisely uncover the mechanism whereby *H. pylori* dysregulates host autophagy.

Keywords: Autophagy; *Helicobacter pylori*; Interleukin-8; Peptic ulcer; Gene expression

INTRODUCTION

Gastric or stomach cancer (GC) has the fifth highest prevalence among malignancies and is the third main reason for annual global death due to cancer (1). In the last few decades, *Helicobacter pylori* (*H. pylori*) infection has been recognized as a primary cause of GC and a significant risk factor for this cancer type as it leads to gastritis that can progress to gastric atrophy and adenocarcinoma, metaplasia, and dysplasia (2, 3). In 1994, this pathogen was categorized by the World Health Organization (WHO) as a

*Corresponding author: Abbas Yadegar, Ph.D, Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tel: +98-21-22432518; +98-9112352488 Fax: +98-21-22432527 Email: a.yadegar@sbmu.ac.ir

class 1 carcinogen and it was reported that almost all GC cases are associated with this bacterial species (4). Epidemiologically, *H. pylori* is capable of colonizing the gastric mucosa in about 50% of the human population in the world; however, the colonization rate varies significantly, being higher in developing than developed countries (5). A recent systematic review and meta-analysis has demonstrated that the prevalence of *H. pylori* infection differs significantly across various areas globally. Data from 32 countries have revealed that the highest prevalence of GC in *H. pylori*-infected patients was found in Japan (90.9%), while the lowest was observed in Sweden (0.07%) (6).

H. pylori is a microaerophilic, extracellular pathogen that infects humans by colonizing on the gastric epithelium surface, leading to chronic infections (7). *H. pylori* infection in many carriers does not cause any significant symptoms. However, it has been documented that prolonged and persistent *H. pylori* colonization greatly enhances the risk of developing various upper gastrointestinal disorders (8). Approximately 10% of infected people are susceptible to developing peptic ulcer disease (PUD). Moreover, 1-3% and <0.1% may develop GC and mucosa-associated lymphoid tissue (MALT) lymphoma, respectively (9).

Clinical manifestations of *H. pylori* infection are mainly dependent on the interplay between the virulence of the bacteria and the genetics of the host (10). The virulence factors of *H. pylori* are categorized into colonization, immune escape, and disease development, which are the three primary pathogenic processes (11). So far, several *H. pylori* putative virulence factors, such as cytotoxin-associated gene A (CagA) in the *cag* pathogenicity island (*cag*PAI), vacuolar cytotoxin A (VacA), urease, high-temperature requirement A (HtrA), gamma-glutamyltranspeptidase (HpGGT), outer inflammatory protein A (OipA), and neutrophil-activating protein (NAP) have been suggested to contribute to its pathogenicity (12). The VacA toxin and CagA oncoprotein are the most recognized virulence factors of *H. pylori*, considerably assisting in GC progression (13).

Evidence has reflected that *H. pylori* could evade autophagy or at least induce defective autophagy, a cellular physiological process that serves a tumor-suppressive function (14, 15). Recent findings have shown an association between the expression of autophagic-related proteins, including Beclin-1 and LC3, and GC prognosis (16). *H. pylori* can inhibit autophagy by activating various cellular signaling pathways, such as Nod1-NF-KB/MAPK-ERK/FOXO4, which allow cell proliferation and tumorigenesis, ultimately leading to GC development (17, 18). Moreover, it has been reported that the disruption of autophagy caused by VacA enhances the accumulation of CagA in cells, inducing gastric carcinogenesis by CagA (19). This survey was undertaken to investigate how various clinical strains of *H. pylori* affect the expression of autophagy-related genes and the production of IL-8 in the human gastric adenocarcinoma (AGS) cell line.

MATERIALS AND METHODS

***H. pylori* clinical strains.** Ten clinical strains of *H. pylori* isolated from Iranian cases were used for cell infection assays. The strains were sourced from five CG patients and five PUD patients. *H. pylori* strains were grown on Brucella agar culture plates (Merck, Germany) containing Campylobacter selective supplement (2.0, 0.05, and 1.0 mg/L of vancomycin, polymyxin, and trimethoprim, respectively), 10% fetal calf serum (FCS), 7% (v/v) horse blood, and amphotericin B (2.5 mg/L). All the plates were then incubated at room temperature under a microaerophilic atmosphere consisting of 10% CO_2, 5% O_2, and 85% N_2 for a time period of 3-5 days. Subsequently, *H. pylori* isolates were biochemically and genetically identified and analyzed for virulence determinants by PCR using specific primers as described elsewhere (20, 21). The features of the *H. pylori* strains included in the present study are summarized in Table 1.

Cell line and culture conditions. The AGS cell line (accession cell no. C10071), procured from the Iranian Biological Resource Center in Tehran (Iran), was cultivated in a complete medium comprising Dulbecco's modified Eagle medium (DMEM; Gibco, CA, USA) consisted of glutamine (2 mM), fetal bovine serum (FBS; 10%, Gibco), penicillin (100 U/ml), and streptomycin (100 µg/ml; Sigma-Aldrich, St. Louis, MO, USA). Finally, the cells were incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C.

Cell treatment and infection. AGS cells were plated in 24-well plates comprising DMEM medium at a density of approximately 5×10^4 cells/well. To achieve the appropriate confluency, the cells were incubated overnight. After attachment, cells at 80%

Table 1. Characterization of the *H. pylori* strains included in this study

No.	Strain ID	Virulence genotype							Patients		
		cagA	*cagL*	*vacA*	*babA2*	*sabA*	*oipA*	*cag*PAI	Clinical outcome	Age, yr	Gender
1	OC217	+	+	s2m2	+	+	Off	Partial	CG	42	F
2	OC485	+	+	s1m1	+	-	On	Intact	CG	54	M
3	OC494	+	+	s1m2	+	+	On	Intact	CG	42	F
4	OC751	+	+	s1m2	-	+	On	Intact	CG	44	F
5	OC840	-	+	s1m2	+	+	Off	Partial	CG	54	M
6	HC114	-	-	s1m2	+	+	On	Partial	PUD	49	F
7	OC557	+	+	s1m2	+	+	On	Partial	PUD	50	F
8	OC814	+	+	s1m1	+	+	On	Intact	PUD	25	F
9	OC913	+	+	s1m2	+	+	On	Intact	PUD	40	M
10	OC939	+	+	s1m2	+	+	On	Intact	PUD	54	M

PUD: peptic ulcer disease; CG: chronic gastritis; male (M), female (F).

confluence were rinsed two time with PBS (pH 7.2) and cultured in serum-free DMEM for 48 h. After harvesting from the Brucella agar plates and suspending in antibiotic-free DMEM medium containing 10% FBS, *H. pylori* strains were utilized to treat the AGS cells. Following infection with the above strains at a multiplicity of infection (MOI) of 100, the cells were incubated at room temperature for 24 h. Finally, the collected AGS cells were utilized for gene expression analysis and cytokine production.

RNA extraction and cDNA synthesis. The extraction of total RNA from infected AGS cells was conducted using the RNeasy Plus Mini Kit (Qiagen, Germany) as per the instruction recommended by the manufacturer with some modifications. The quality of extracted RNA and its quantity were measured by observing the distinct bands of ribosomal RNA (rRNA) on 2% agarose gel electrophoresis by use of a Thermo Scientific NanoDrop spectrophotometer (ND-1000, USA), with a ratio of A260 to 280. The conversion of purified RNA samples to cDNA was carried out by using a RT-kit (BioFACT, South Korea) in accordance with the protocol recommended by the manufacturer. The samples were maintained at the temperature of -80°C until gene expression analysis.

Quantitative real-time PCR. The expression of *atg5, atg12, atg16L1, LC3B,* and *beclin-1,* autophagy-related genes, was determined by RT-qPCR assay in *H. pylori*-infected AGS cells using specific primers (Table 2). Amplification was performed with 2× real-time PCR master mix (BioFACT) by employing a Rotor-Gene Q (Qiagen) real-time PCR system. A melting curve was generated immediately following amplification to verify the specificity of PCR for each product. β-actin served as the internal control, and relative alterations in mRNA levels were evaluated by the comparative cycle threshold (Ct) method. All assays were performed in triplicate.

Cytokine detection by ELISA. Human IL-8 uncoated ELISA Kit (Invitrogen TM, Thermo Fisher Scientific) was used to measure the production of IL-8 cytokine in the supernatant of AGS cells. This process was conducted in accordance with the instructions provided by the manufacturer with minor medications. All samples underwent testing in triplicate.

Statistical analysis. GraphPad Prism 8.0 (CA, United States) was employed to calculate gene expression alterations and cytokine production. To assess differences between the study groups, we conducted a one-way analysis of variance (ANOVA), together with Tukey's post hoc test. Results were presented as the mean ± standard error from a minimum of three experiments. Differences reaching $P \leq 0.05$ were considered statistically significant.

RESULTS

Characteristics of *H. pylori* strains. In current study, ten *H. pylori* strains isolated from CG (n=5) and PUD (n=5) patients were selected (Table 1). The strains were obtained from both men and women with

Table 2. The oligonucleotide sequences used in this study

Target gene	Oligonucleotide sequences (5' 3')	Tm (ºC)	Amplicon (bp)	Ref.
atg12	F: AGTAGAGCGAACACGAACCATCC	57.1	110	(22)
	R: AAGGAGCAAAGGACTGATTCACATA	54.4		
atg5	F: AGAAGCTGTTTCGTCCTGTGG	54.4	152	(23)
	R: AGGTGTTTCCAACATTGGCTC	52.4		
atg16L1	F: ATGCGCGGATTGTCTCAGG	53.2	138	(24)
	R: GTCCACTCATTACACATTGCTCT	53.5		
LC3B	F: AACGGGCTGTGTGAGAAAAC	51.8	84	(25)
	R: AGTGAGGACTTTGGGTGTGG	53.8		
beclin-1	F: CTGAGGGATGGAAGGGTC	52.6	159	(22)
	R: TGGGCTGTGGTAAGTAATG	48.9		
β-actin	F: ATGTGGCCGAGGACTTTGATT	52.4	107	(26)
	R: AGTGGGGTGGCTTTTAGGATG	54.4		

a mean age of 46.5 years. Almost all *H. pylori* strains were *cagA*- and *cagL*-positive (80% and 90%, respectively), and more than half of the strains contained an intact *cag*PAI. Among the strains, the *vacA* allele s1m2 was the most common *vacA* genotype. All the strains from PUD patients were detected as *babA2*- and *sabA*-positive (100%), whereas 90% of the strains from CG patients carried these adhesion-associated genes. Interestingly, all the strains from PUD patients were examined for harboring *oipA* "on" motifs.

Expression of *atg5, atg12,* and *atg16L1* level in *H. pylori*-infected AGS cells. To assess whether the clinical *H. pylori* strains could interfere with autophagy, we measured the gene expression of *atg5, atg12,* and *atg16L1* in infected AGS cells. All *H. pylori* strains exhibited the capability of upregulating the expression of above-mentioned genes in AGS cells. However, the gene upregulation was detected as statistically significant upon exposure to some *H. pylori* strains, mostly the strains from PUD patients. Fig. 1 illustrates the expression levels of *atg5, atg12,* and *atg16L1* in *H. pylori*-infected AGS cells relative to uninfected AGS cells that served as the control group. The exact fold changes and 95% confidence intervals of target genes in AGS cells infected with *H. pylori* strains are presented in Table 1.

Expression level of *LC3B* in *H. pylori*-infected AGS cells. The expression level of *LC3B*, a key autophagy marker, was examined. The *LC3B* expression level was elevated by all strains of *H. pylori* in comparison to uninfected AGS cells (Fig. 2). However, *H. pylori* strains from PUD patients, relative to the strains from GC patients, revealed a stronger effect on the gene upregulation of *LC3B.*

Expression level of *beclin-1* in *H. pylori*-infected AGS cells. In our investigation, the *beclin-1* mRNA expression level was determined due to the importance of this protein in autophagy. As shown in Fig. 3, the *beclin-1* gene was downregulated in all *H. pylori*-infected AGS cells. However, *H. pylori* strains from PUD patients markedly reduced the expression of *beclin-1* level in comparison to strains from CG patients.

IL-8 production in *H. pylori*-infected AGS cells. We examined if *H. pylori* strains stimulate the production of IL-8 in gastric epithelial cells by infecting AGS cells with 10 clinical strains obtained from both CG and PUD cases. As shown in Fig. 4, all *H. pylori* strains elevated the IL-8 production in AGS cells. However, no major differences were detected between strains from CG and PUD cases. Moreover, statistically significant difference between higher IL-8 levels and greater upregulation or downregulation of autophagy genes were observed to be non-significant.

DISCUSSION

The host immune system against bacterial pathogens needs the coordination of multiple innate immune signaling pathways (27). The recognition of molecular patterns associated with pathogens by the innate immune system receptors could trigger vari-

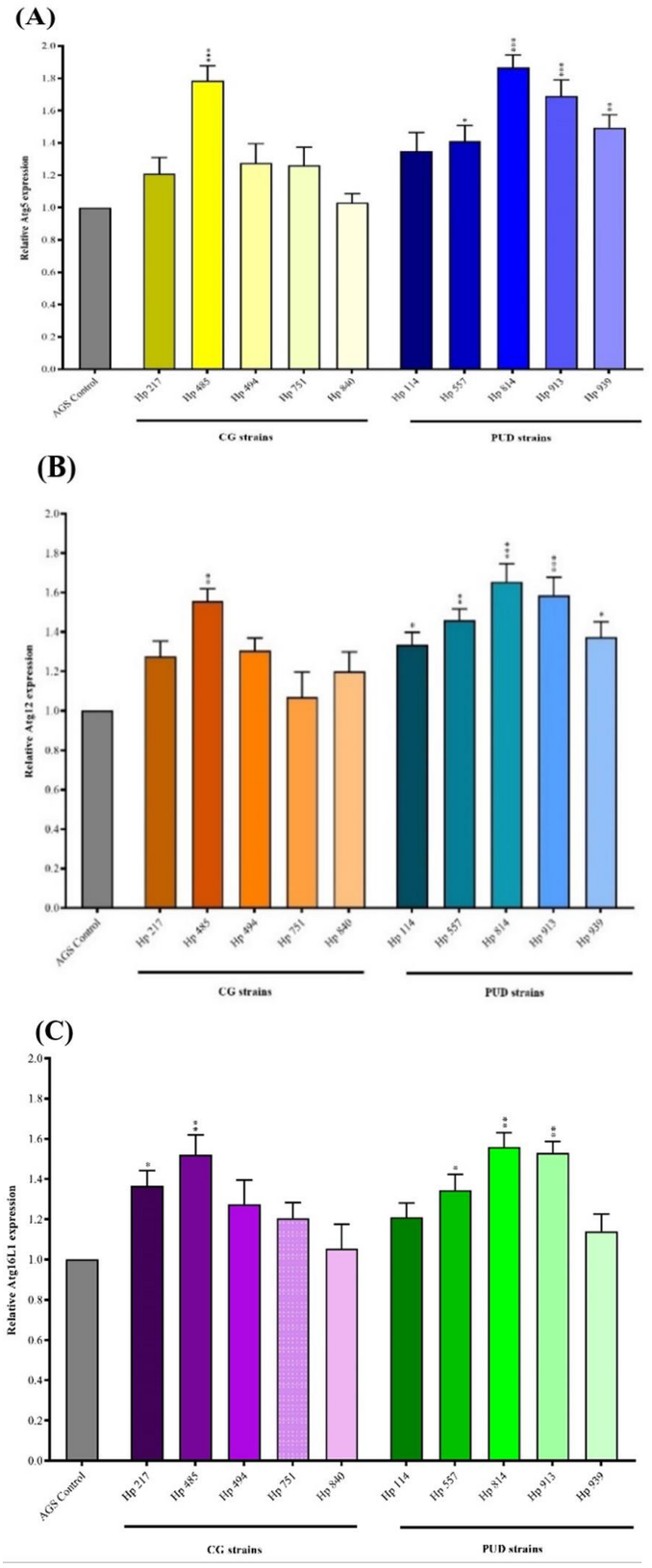

Fig. 1. The relative gene expression of *atg5* (A), *atg12* (B), and *atg16L1* (C) in AGS cells infected with clinical *H. pylori* strains isolated from patients with chronic gastritis (CG) and peptic ulcer disease (PUD) after 24 h of exposure. Gene expression data were normalized to β-actin as the reference gene. Data are presented as mean ± SD from three independent experiments. A P value of <0.05 was considered significant (*P <0.05; **P <0.01; ***P <0.001; ****P <0.0001) by one-way ANOVA statistical analysis.

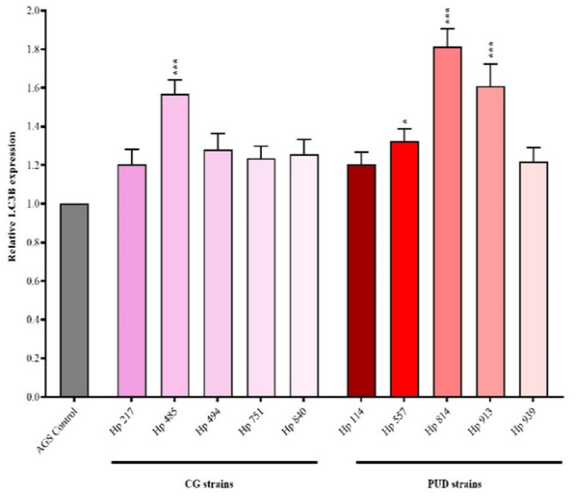

Fig. 2. The relative gene expression of *LC3B* in AGS cells infected with clinical *H. pylori* strains isolated from patients with chronic gastritis (CG) and peptic ulcer disease (PUD) after 24 h of exposure. Gene expression data were normalized to β-actin as the reference gene. Data are presented as mean ± SD from three independent experiments. A P value of <0.05 was considered significant (*P <0.05; **P <0.01; ***P <0.001; ****P <0.0001) by one-way ANOVA statistical analysis.

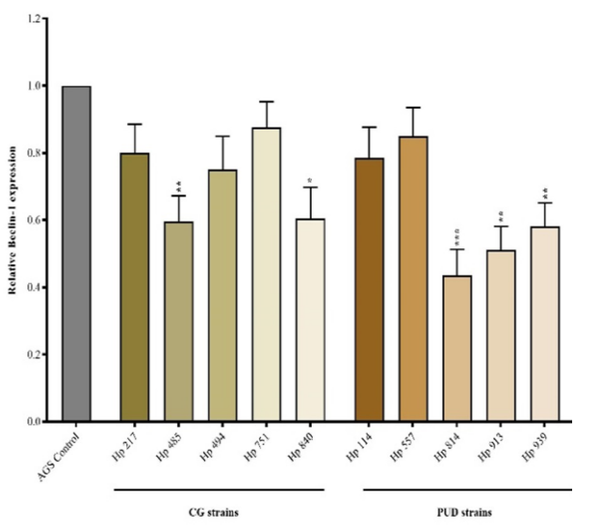

Fig. 3. The relative gene expression of *beclin-1* in AGS cells infected with clinical *H. pylori* strains isolated from patients with chronic gastritis (CG) and peptic ulcer disease (PUD) after 24 h of exposure. Gene expression data were normalized to β-actin as the reference gene. Data are presented as mean ± SD from three independent experiments. A P value of <0.05 was considered significant (*P <0.05; **P <0.01; ***P <0.001; ****P <0.0001) by one-way ANOVA statistical analysis.

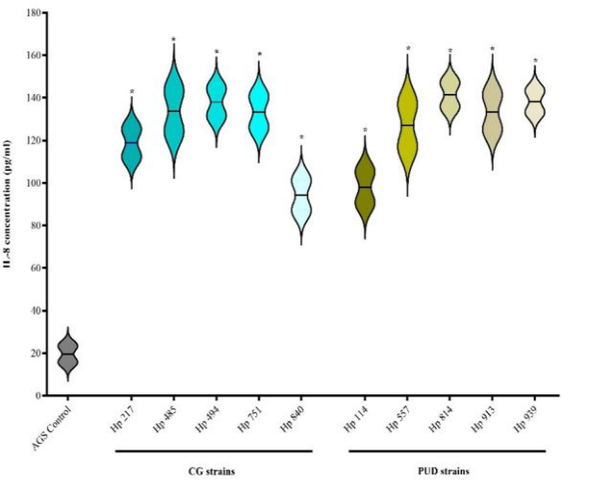

Fig. 4. The production of proinflammatory cytokine IL-8 measured by ELISA in AGS cells infected with clinical *H. pylori* strains isolated from patients with chronic gastritis (CG) and peptic ulcer disease (PUD) after 24 h of exposure. Data are presented as mean ± SD from three independent experiments. A P value of <0.05 was considered significant (*P <0.05; **P <0.01; ***P <0.001; ****P <0.0001) by one-way ANOVA statistical analysis.

ous intracellular signaling pathways, activating host antibacterial defense mechanisms to eliminate the microbial agent (28). Autophagy has long been considered to be one of the dynamic processes located downstream of the innate immune-associated receptors and to have a critical role in resisting various microbial pathogens (29). Although various microbial pathogens are targeted and eradicated by the autophagy machinery, some bacterial pathogens have been identified to evade this defense mechanism or even manipulate the autophagy process to enhance their survival (30, 31). For instance, intracellular pathogens *Yersinia enterocolitica*, *Francisella tularensis*, and *Orientia tsutsugamushi* have been described to evade autophagy; however, host features and the exact bacterial virulence factors that contribute to their evasion have not been thoroughly clarified (32-34). Moreover, the dysregulation of autophagy machinery during the crosstalk of microorganisms and the host can not only develop numerous types of cancers but also trigger tumor cell survival (35). Accordingly, the role of autophagy induced by *Porphyromonas gingivalis* in oral cancer cell proliferation and by *Fusobacterium nucleatum* in colorectal cancer pathogenesis has previously been described (36, 37).

Given the recent reports highlighting the interac-

tion between autophagy and *H. pylori* infection, our study attempted to investigate whether clinical *H. pylori* strains from CG and PUD could regulate the expression of genes related to autophagy proteins. The main observation was that overexpression in autophagy-related genes, except *beclin-1*, could happen in relation to *H. pylori* infection. Our findings align with those of Courtois et al. who found that the expression of genes involved in the autophagy process and the number of autophagosomes increased, indicating that *H. pylori* infection triggers autophagy and could be implicated in developing gastric cancer stem cells (38). During *H. pylori* infection, autophagy could be induced and activated, mainly due to the outer membrane vesicles (OMVs), VacA, and HP0175 of the bacterium (39). The HP0175 antigen has been demonstrated to upregulate the expression of genes associated with autophagy, independent of functional VacA during acute infection (40). However, it has formerly been explored that many intracellular pathogens could inhibit autophagy by downregulating autophagy-related genes (41). In contrast, Tanaka et al. reported the downregulation of ATG16L1, ATG5, ATG4D, and ATG9A genes, as the main autophagy machinery factors, by using high-throughput microarray analysis (42). Altogether, these observations indicate that infection with *H. pylori* may result in partially incomplete autophagy without content degradation, meaning that autophagy could be induced but not completely activated. In other words, a proportion of autophagy-related genes are upregulated and others are downregulated. The reason for the induction of this partial autophagy could be the accumulation of malfunctioning autophagosomes containing *H. pylori*. This process limits exposure to antibiotics and promotes bacterium's survival within cells, potentially providing a favorable environment for *H. pylori* within gastric epithelial cells (43, 44). Additionally, according to a study by Xie et al., *H. pylori* infection increases autophagy in the early stages of infection, but in later stages it is able to inhibit the autophagy pathway by reducing Rad51 expression (45). The discrepancy among these studies could be attributed to difference in infection phases. While the current study focused and investigated early gene expression responses, other studies focused on autophagy flux during long-term infection, where inhibition is more likely to occur. To confirm this hypothesis, additional investigations are needed to clarify the exact mechanism and functional role of *H. pylori* infection in the expression of autophagy-related genes during its pathogenesis, particularly in inflamed and cancerous tissues.

Our current observations reveal that *H. pylori* strains from PUD patients were more likely to upregulate the core component autophagy-related genes, whereas CG strains were less able to stimulate. The diversity of clinical strains has a critical role in characterizing host-pathogen interactions. Considering that variations in virulence factors, genetic patterns, and host immune response stimulation could lead to distinct activation of autophagy and inflammatory pathways. This heterogenicity may explain the wide spectrum of *H. pylori* infection-associated clinical outcomes (46, 47). It has previously been proven that long-term *H. pylori* infection could prevent not only autophagy but also apoptosis processes via the Nod1-NF-kB/ MAPK-ERK/FOXO4 signaling pathway, thus resulting in the onset of GC (48, 49). On the other hand, recent surveys exhibit that the process of autophagy can be activated and induced by VacA, CagA, HpGGT, and HP0175, the virulence factors of *H. pylori*, in the epithelial cells of the gastric mucosa (40, 50-52). Accordingly, these outcomes demonstrate that the acute *H. pylori* infection can induce autophagosome formation, while the downregulation of autophagy-related proteins and their downstream genes induced by *H. pylori* could inhibit the autophagy process and enhance the growth and proliferation of tumor cells, consequently developing GC. Notably, the exact mechanisms of autophagy controlled by *H. pylori* infection are rather complicated, and further investigations are needed to meticulously explicate the involved factors and mechanisms. Huang et al. previously described that *beclin-1* expression was notably downregulated in GC linked to CagA-positive *H. pylori* infection (53), which supports the results of our study.

Beclin-1-mediated autophagy has been described to suppress myr-Akt1-driven tumorigenesis and could hence decrease tumor cellular proliferation and increase tumor cell death (54). Moreover, *beclin-1* overexpression has been shown to obviously mitigate the capability of gastric cancerous cells in migrating and invading, probably through vascular endothelial growth factor (VEGF) dysregulation (55). These data suggest that *H. pylori* colonization could have a link with autophagy suppression in the metastasis and neoplasia of GC.

CONCLUSION

According to results of current study, the mechanisms of *H. pylori*-mediated autophagy dysregulation in the gastric epithelial cells are extremely complicated. Infection with *H. pylori* could result in changes in autophagy-related gene expression through multifactor signaling pathways. Several protein network regulation systems have been recognized to play their respective roles in the dysregulation of autophagy in human host cells and interact with each other. Once an autophagy-related gene is dysregulated or abnormally downregulated, it can trigger a chain of cellular processes and damages, ultimately developing gastric cancer. Hence, successful treatment of *H. pylori* infection targeting autophagy-related pathways could be considered a novel strategy, signifying a new approach to treat *H. pylori*-induced GC that engages different host-microbial interactions. However, it is necessary to note that current results on autophagy dysregulation are based on mRNA expression levels and functional validation of autophagic flux was not performed. Furthermore, these in vitro investigations may not clearly reflect in vivo or clinical conditions.

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