World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v22.i15.3978

World J Gastroenterol 2016 April 21; 22(15): 3978-3991
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Mir-30d increases intracellular survival of *Helicobacter pylori* through inhibition of autophagy pathway

Xiao-Jun Yang, Ruo-Huang Si, Yu-He Liang, Bing-Qiang Ma, Ze-Bin Jiang, Bin Wang, Peng Gao

Xiao-Jun Yang, Ruo-Huang Si, Bing-Qiang Ma, Ze-Bin Jiang, Bin Wang, Peng Gao, Department of General Surgery, Gansu Provincial Hospital, Lanzhou 730000, Gansu Province, China

Yu-He Liang, Department of General Surgery, The People's Hospital of Baoji City, Baoji 721000, Shaanxi Province, China

Author contributions: Yang XJ, Si RH and Liang YH contributed equally to this work; Yang XJ and Liang YH carried out cell culture, transfection and the molecular genetic studies, drafted the manuscript; Si RH carried out the RT-PCR; Ma BQ and Jiang ZB participated in the GFP-LC3 Plasmid Transfection and Flow cytometry; Wang B performed the statistical analysis; and Gao P conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

Supported by the National Natural Science Fund from China, No. 81260326.

Institutional review board statement: This study was reviewed and approved by Gansu Provincial Hospital Institutional review board.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional animal care and use committee of Gansu Provincial Hospital.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: No additional data are available.

Correspondence to: Peng Gao, MD, Professor, Chief, Department of General Surgery, Gansu Provincial Hospital, No. 204 Donggang West Road, Lanzhou 730000, Gansu Province, China. gaopenglz@aliyun.com
Telephone: +86-931-8281015
Fax: +86-931-8266957

Received: December 7, 2015
Peer-review started: December 10, 2015
First decision: December 30, 2015
Revised: January 14, 2016
Accepted: February 20, 2016
Article in press: February 22, 2016
Published online: April 21, 2016

Abstract

AIM: To determine if mir-30d inhibits the autophagy response to *Helicobacter pylori* (*H. pylori*) invasion and increases *H. pylori* intracellular survival.

METHODS: The expression of mir-30d was detected by quantitative polymerase chain reaction (PCR), and autophagy level was examined by transmission electron microscopy, western blot, and GFP-LC3 puncta assay in human AGS cells and GES-1 cells. Luciferase reporter assay was applied to confirm the specificity of mir-30d regulation on the expression of several core molecules involved in autophagy pathway. The expression of multiple core proteins were analyzed at both the mRNA and protein level, and the intracellular survival of *H. pylori* after different treatments was detected by gentamicin protection assay.

RESULTS: Autophagy level was increased in AGS and GES-1 cells in response to *H. pylori* infection, which was accompanied by upregulation of mir-30d expression ($P < 0.05$, *vs* no *H. pylori* infection). In the two gastric epithelial cell lines, mimic mir-30d was found to repress the autophagy process, whereas mir-30d inhibitor increased autophagy response

to *H. pylori* invasion. mir-30d mimic decreased the luciferase activity of wild type reporter plasmids carrying the 3′ untranslated region (UTR) of all five tested genes (*ATG2B*, *ATG5*, *ATG12*, *BECN1*, and *BNIP3L*), whereas it had no effect on the mutant reporter plasmids. These five genes are core genes of autophagy pathway, and their expression was reduced significantly after mir-30d mimic transfection ($P < 0.05$, *vs* control cells without mir-30d mimic treatment). Mir-30d mimic transfection and direct inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

CONCLUSION: Mir-30d increases intracellular survival of *H. pylori* in gastric epithelial cells through inhibition of multiple core proteins in the autophagy pathway.

Key words: mir-30d; *Helicobacter pylori*; Autophagy; Gene expression; Gastric cancer

Core tip: In this study, we tested a hypothesis that mir-30d could repress autophagy in response to *Helicobacter pylori* (*H. pylori*) invasion by directly targeting multiple core genes of the autophagy pathway, including ATG2B, ATG5, ATG12, BECN1 and BNIP3L in gastric epithelial cells. Inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells, and the repression of autophagy by mir-30d may help the intracellular *H. pylori* to evade autophagic clearance. These findings provide a novel mechanism for elucidating persistent *H. pylori* infection and provide a promising target for gastric cancer prevention.

Yang XJ, Si RH, Liang YH, Ma BQ, Jiang ZB, Wang B, Gao P. Mir-30d increases intracellular survival of *Helicobacter pylori* through inhibition of autophagy pathway. *World J Gastroenterol* 2016; 22(15): 3978-3991 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/3978.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.3978

INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death in the world, and almost two-thirds of the cases occur in Asian countries, especially China and Japan[1,2]. The prognosis of gastric cancer is generally rather poor, and, therefore, prevention is a better choice than cure for patients with gastric cancer.

Helicobacter pylori (*H. pylori*) is a class I carcinogen, appointed by the International Agency for Research on Cancer in 1994 due to its strong correlation with gastric cancer in humans[3]. One reason for *H.pylori*'s high resistance to biomedical therapy may be its residence inside host cells[4,5]. Although regarded generally as an extracellular pathogen, the intracellular survival of *H. pylori* in both gastric epithelial cells and immunocytes allows it to escape from the host immune response and resist destruction from membrane-impermeable antibiotics[6], leading to persistence in the stomach. Up to now, the detailed molecular mechanisms by which *H. pylori* escape host cell machineries for intracellular survival are remains obscure.

Autophagy is present in mammalian cells at a low basal level. As an evolutionarily conserved cellular activity, it delivers organelles and cellular materials to the lysosome for degradation within double-membraned vacuoles, called autophagosomes[7,8]. Autophagy is considered one of the innate immune effectors against intracellular bacterial infection (*e.g.*, *Streptococcus pyogenes*)[9,10]. Autophagic proteins act as cytosolic sensors to rapidly launch the autophagic pathway when the innate defense system recognizes invasive bacterial pathogens[11]. However, some intracellular pathogens use highly evolved machinery to deceive autophagic recognition, manipulate the autophagic pathway, and reconstruct the autophagosomal compartment for their own survival[12]. Over the last decade, many studies have reported that *H. pylori* infection can induce macroautophagy and that *H. pylori* may evade the autophagic machinery through downregulating the expression of autophagic proteins[6,13-15].

Recently, interest in the study of mir-30 has been growing. The mir-30 microRNA family is extensively expressed in multiple tissues and cell types[16,17]. It has been shown to be involved in a wide range of physiological activities in normal tissues and cancer tissues, including cell differentiation, development, proliferation, apoptosis, senescence, and cancer metastasis[18-22]. mir-30 expression is amplified in more than 30% of human epithelial tumors, including gastric cancer[15,23,24]. There is increasing evidence that mir-30 is a novel oncomir and understanding the mechanism underlying mir-30 function in tumorigenesis would be helpful for developing targeted cancer therapy against this miRNA family. Previously, we demonstrated that mir-30d regulated cellular autophagy by directly targeting multiple genes in the autophagy pathway[25]. Consistent with our finding, another mir-30 family member, mir-30a was found to regulate autophagy *via* repressing BECN1 expression in tumor cells[26,27]. In addition, compromised autophagy by mir-30b upregulation might benefit the intracellular survival of *H. pylori*[15]. These results shed light on the potential role of miRNAs on autophagy regulation during gastric tumorigenesis.

Here, we continue our investigation on mir-30d and *H.pylori* and suggest that mir-30d downregulated the expression of key autophagy genes, including ATG2B, ATG5, ATG12, BECN1 and BNIP3L, and inhibited the autophagy response to *H.pylori* invasion of

gastric epithelial cells, resulting in increased *H. pylori* intracellular survival.

MATERIALS AND METHODS

Plasmids

The green fluorescent protein (GFP)-LC3 and psiCHECK-2 vectors were purchased from Addgene (Cambridge, MA, United States) and Promega (Madison, WI, United States), respectively.

Antibodies and reagents

Antibodies against light chain 3 B (LC3B), autophagy related (ATG)2B, ATG5, ATG12, beclin 1 (BECN1), and BNip3-like protein (BNIP3L) were obtained from Cell Signaling Technology (CST, Beverly, MA, United States). 3-methyladenine (3-MA, M9281) and rapamycin (Rapa, R8781) were purchased from Sigma (St. Louis, MO, United States).

Cell lines and H. pylori strains

AGS cells (a human gastric adenocarcinoma cell-line) were obtained from American Type Culture Collection (Manassas, VA, United States) and cultured in F12 media (Gibco, Carlsbad, CA, United States). Human gastric mucosal epithelial cell line GES-1 (Purchased from Cell bank of Xiangya Medical School, Central South University, Hunan, China) was cultured in Roswell Park Memorial Institute (RPMI)1640 (Cellgro, Manassas, VA, United States) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, United States), and 100 U/mL penicillin/streptomycin (Gibco, 15140-122) in a humidified incubator at 37 ℃ with 5% CO_2. For autophagy induction, cells were either treated with 200 nM rapamycin (Sigma) supplemented in complete medium or serum starved with Hank's buffer (Stemcell Technologies, Vancouver, Canada), both at 37 ℃ for 4 h. The wild-type *H. pylori* strain 26695(700392) was obtained from American Type Culture Collection and cultured as previously described.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed using a high capacity RNA-cDNA kit (Applied Biosystems, Carlsbad, CA, United States). cDNA was quantified on an ABI Prism 7900 sequence detection system (Applied Biosystems). Polymerase chain reaction was performed using Power SYBR Green polymerase chain reaction (PCR) master mix (Applied Biosystems).

Western blotting

Cells were lysed in mammalian protein extraction reagent (Pierce, Rockford, IL, United States) with protease inhibitor cocktail (Sigma). After centrifugation at 5000 g for 15 min at 4 ℃, the protein concentration was measured with bicinchoninic acid (BCA) protein assay kit (Pierce, 23227). Fifteen micrograms of total protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, United States). Membranes were blocked in 5% non-fat milk (Bio-Rad, Hercules, CA, United States) and then incubated with the following primary antibodies: anti-ATG2B, anti-ATG5, anti-ATG12, anti-BECN1, anti-BNIP3L, and anti-LC3B. After incubation with a secondary antibody conjugated with horseradish peroxidase (HRP) (Amersham Biosciences, Chalfont St. Giles, United Kingdom) together with an HRP-conjugated primary antibody for b-actin (Sigma), immunoreactive proteins were visualized using the LumiGLO chemiluminescent substrate (Cell Signaling). Densitometric analyses were performed using Scion Image software.

GFP-LC3 plasmid transfection

Cells were seeded onto six-well plates and transfected with a GFP-LC3 expression plasmid at approximately 45%-55% confluence using the Lipofectamine RNAiMAX transfection reagent (Invitrogen). After 24 h, the cells were infected with or without *H. pylori* for 24 h. For observation, cells were fixed with 4% formaldehyde for 15 min and then washed twice in cold phosphate-buffered saline (PBS). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Transfection of mir-30d mimic and inhibitor oligonucleotides

Pre-mir miRNA precursor and control oligos were purchased from Ambion (Foster City, CA, United States), and miRCURY LNA miRNA inhibitors and control oligos were purchased from Exiqon (Vedbaek, Denmark). Transfections were performed using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) and then cells were incubated in the medium containing the transfection mixture for 24-48 h.

Luciferase reporter assay

Cells were plated on a 24-well plate 24 h before transfection at 50% confluence. miRNA mimics (30 nmol/L, Ambion) were transfected using Lipofectamine RNAiMAX. Twenty-four hours post-transfection, 0.125 μg of reporter vector was transfected using FuGENE6 transfection reagent (Roche, Basel, Switzerland). Forty-eight hours after reporter vector transfection, cells were harvested, and reporter assays were performed using a dual luciferase reporter assay system (Promega).

Transmission electron microscopy

AGS and GES-1 cells were digested with 0.25% trypsinase and rinsed twice with PBS. They were then collected, fixed in 2% paraformaldehyde, 0.1%

glutaraldehyde in 0.1 mol/L sodium cacodylate for 2 h, postfixed with 1% OsO_4 for 1.5 h, washed, and stained for 1 h in 3% aqueous uranyl acetate. The samples were then washed again, dehydrated with graded alcohol, embedded in Epon-Araldite resin (Canemco, Quebec, Canada), and then cut into 0.05 μm thick sections on an ultramicrotome. The cells were observed under JEM-1230 (Jeol Ltd., Tokyo, Japan) electron microscopy.

Gentamicin protection assay

After bacteria infection, the GES-1 and bacterium co-culture was washed three times with 1 mL of warm PBS per well to remove nonadherent bacteria. To determine the colony-forming unit (CFU) count corresponding to intracellular bacteria, the GES-1 cell monolayers were treated with gentamicin (100 mg/mL; Sigma, G1272) at 37 ℃ in 5% CO_2 for 1 h, washed three times with warm PBS, and then incubated with 1 mL of 0.5% saponin (Sigma, 47036) in PBS at 37 ℃ for 15 min. The treated monolayers were resuspended thoroughly, diluted, and plated on serum agar. To determine the total CFU corresponding to host associated bacteria, the infected monolayers were incubated with 1 mL of 0.5% saponin in PBS at 37 ℃ for 15 min without prior treatment with gentamicin. The resulting suspensions were diluted and plated as described above. Both the CFU of intracellular bacteria and the total CFU of cell-associated bacteria were given as CFU per well of GES-1 cells.

Bioinformatic analysis

miRNA and mRNA expression microarray data were retrieved from a public accessible database, Cell Miner. http://discover.nci.nih.gov/cellminer/. Gene set enrichment analysis (GSEA) algorithm was used to identify the pathways that were significantly enriched between mir-30d low and high tumor cells. http://www. broadinstitute.org/gsea/index.jsp. TargetScan algorithm was used to predict mir-30d targets. http://www.targetscan.org.

RESULTS

H. pylori infection increased autophagy increased and upregulated mir-30d in AGS and GES-1 cell lines in response to

To measure autophagy induction during *H. pylori* infection, a GFP-LC3 fusion protein expression reporter was used in the assay. Upon autophagy induction, LC3-I, one form of the microtubule-associated protein light chain 3 (LC3), converts to another form LC3-Ⅱ. LC3-Ⅱ is accumulated in the autophagosomal membranes, and its amount is correlates to the number of autophagosomes and may serve as a marker for autophagosome formation. Autophagy induction was evaluated by measuring the quantity of GFP-LC3 puncta formed in the tested cell. AGS and GES-1 cells were transfected with GFP-LC3 vector and infected with or without *H. pylori* for autophagy analysis. Under fluorescence microscopy observation (Figure 1A), the GFP-LC3 puncta was significantly increased in *H. pylori* infected AGS and GES-1 cells (compared to the control cells without infection) after 24 h infection. This finding indicated that *H. pylori* infection may induce LC3-Ⅱ production and autophagosome formation.

Meanwhile, a typical autophagosome, double-limiting membrane, was detectable in the autophagosome (black arrowheads) and autophagolysosome (white arrowheads) examined by transmission electron microscopy (TEM) (Figure 1B). Ultrastructural image analysis showed the presence of double-membrane autophagic vesicles containing *H. pylori* in the cytoplasm of AGS cells. The number of autophagic vacuoles (AV), including autophagosomes and autophagolysosomes in *H. pylori* infected AGS cells, was increased (Figure 1B). Similar results were obtained from GES-1 cells (Figure 1B).

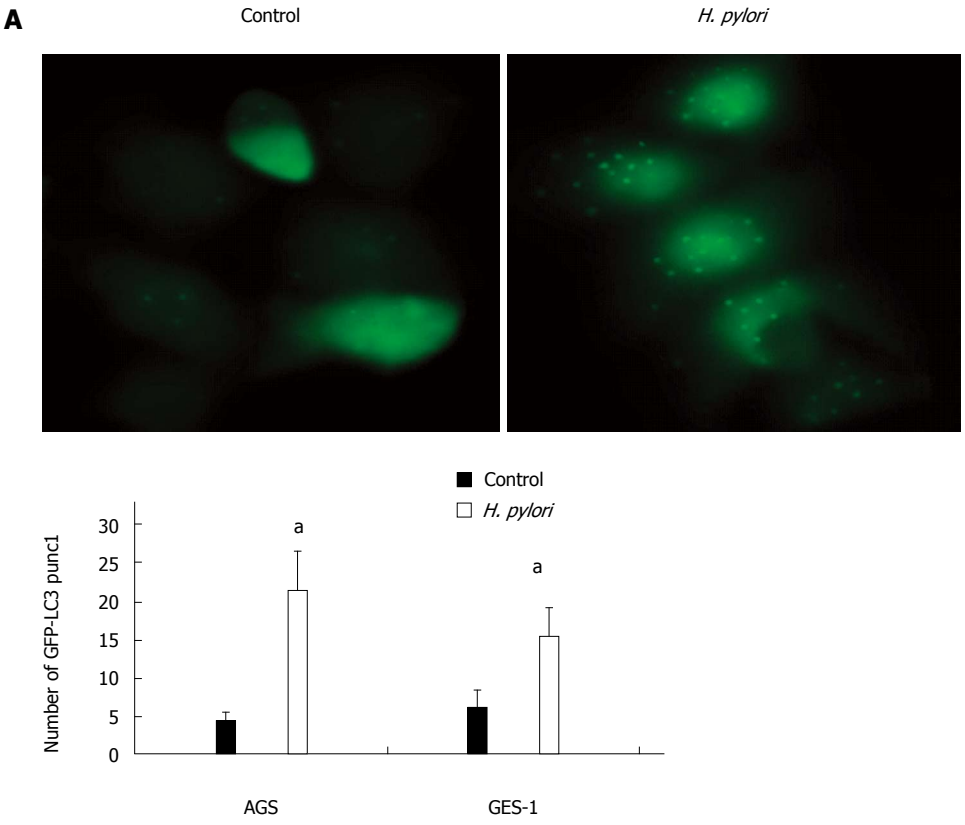
To further confirm this finding, western blots for LC3B protein were applied to analyze the conversion of LC3B-I to LC3B-Ⅱ. At 12 h and 24 h after *H. pylori* infection, LC3B-Ⅱ protein level was significantly increased in AGS cells when compared with non-infected cells. A similar pattern was observed in GES-1 cells as well (Figure 1C). The expression of mir-30d in *H. pylori* infected cells was analyzed by quantitative real-time PCR, and the results showed that the expression of mir-30d was obviously increased at 12 h and 24 h after being infected with *H. pylori* in both AGS and GES-1 cell lines ($P < 0.05$, *H. pylori* infected cells versus without *H. pylori* infected cells) (Figure 1D).

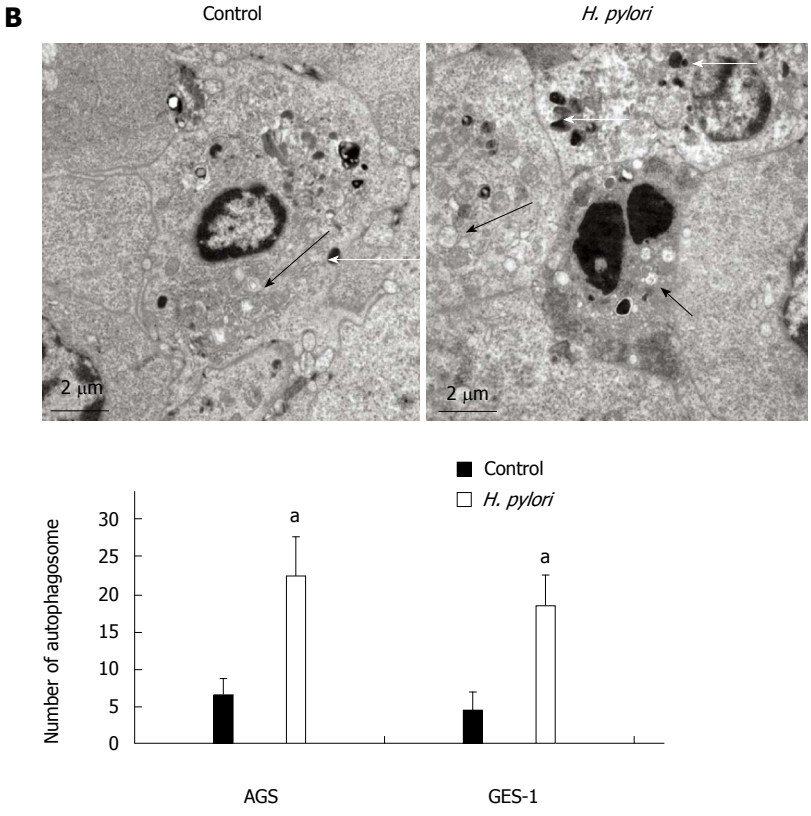
Taken together, these data demonstrate that *H.pylori* infection increased the conversion of LC3B-I to LC3B-Ⅱ (hence higher autophagosome formation), introduced a complete autophagic response, and upregulated mir-30d expression in AGS and GES-1 cell lines.

Transfection of mir-30d mimic in AGS and GES-1 cell lines downregulates autophagy after H. pylori *infection*

To determine whether mir-30d has a role in the negative regulation of autophagy during *H. pylori* infection, a mir-30d mimic was transfected into AGS and GES-1 cell lines for 24 h and then infected with *H. pylori*. The effect of the mir-30d mimic on autophagy was examined in both cell lines by western blotting, GFP-LC3 puncta assay, and TEM at 24 h post-*H. pylori* infection. Figure 2A showed that the expression of mir-30d was significantly increased in AGS cell lines either with or without *H. pylori* infection at 48 h after mir-30d mimic transfection ($P < 0.05$, mimics *vs* control). The same results were also found in GES-1 cells ($P < 0.05$ mimic transfected cells *vs* no mimic transfected control cells).

The results of western blotting revealed that


A
Control
H. pylori
Control
H. pylori
Number of GFP-LC3 punc1
30
25
20
15
10
5
0
a
a
AGS
GES-1


B
Control
H. pylori
2 μm
2 μm
Control
H. pylori
Number of autophagosome
30
25
20
15
10
5
0
a
a
AGS
GES-1

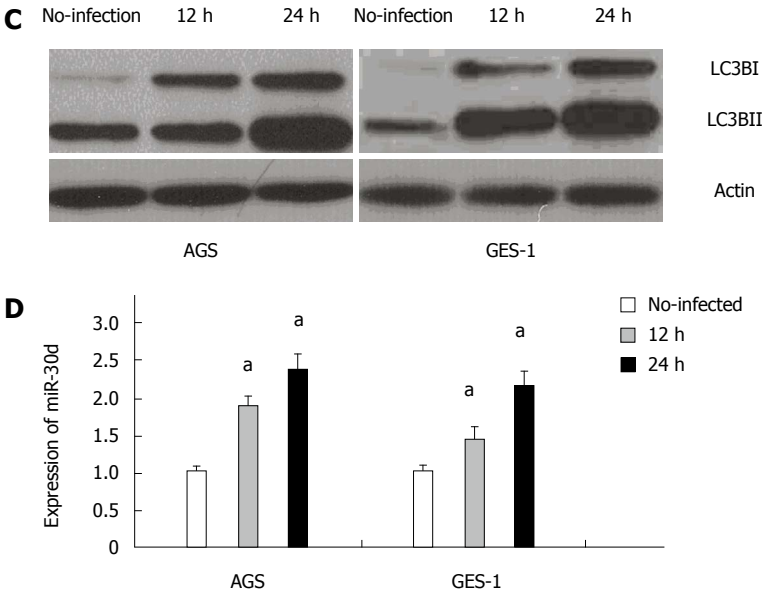


Figure 1 Autophagy and mir-30d are upregulated in AGS and GES-1 cell lines in response to *Helicobacter pylori* infection. A: GFP-LC3 puncta were observed in AGS cells with or without 24 h *Helicobacter pylori* (*H. pylori*) infection. Quantification of the number of GFP-LC3 puncta in AGS and GES-1 cells presented as mean ± SD, $^{a}P < 0.05$ control *vs H. pylori* infection; B: The autophagosomes and autophagolysosomes at 24 h after *H. pylori* infection assayed by transmission emission microscopy (TEM), shown are a typical autophagosome (black arrowheads) and autophagolysosome (white arrowheads). Quantification of GFP-LC3 puncta in AGS and GES-1 cells shown as mean ± SD, $^{a}P < 0.05$ *vs* control; C: The protein levels of light chain (LC)3B-I and LC3B-Ⅱ at 12 h and 24 h after infection with *H. pylori* analyzed by western blot; D: Analysis of the expression of mir-30d at 12 h and 24 h after infection with *H. pylori* in both cell lines by quantitative polymerase chain reaction (q-PCR). Results shown as mean ± SD, $^{a}P < 0.05$ *vs* control.

autophagy was enhanced, as evidenced by increased LC3B-Ⅱ expression, in both two cell lines during *H. pylori* infection, but transfection of mir-30d mimic significantly downregulated autophagy activity *(i.e.*, attenuated LC3B-Ⅱ conversion, Figure 2B).

In the GFP-LC3 puncta assay, GFP-LC3 plasmid and mir-30d mimic/control mimic were co-transfected into AGS and GES-1 cells using the lipofectamine RNAiMAX transfection reagent for 24 h and then infected with *H. pylori*. The treated cells were imaged under confocal laser-scanning microscope 24 h after *H. pylori* infection. The results showed that GFP-LC3 puncta were significantly increased in AGS and GES-1 cells infected with *H. pylori* compared to non- infected cells at 24 h after *H. pylori* infection, but transfection of mir-30d mimic significantly decreased GFP-LC3 positive puncta in AGS and GES-1 cells ($P < 0.05$, mimics *vs* control; Figure 2C).

Meanwhile, autophagosome and autophagolysosome were examined by TEM. The number of autophagic vacuoles (AV), including autophagosomes and autophagolysosomes, was increased in *H. pylori* infected AGS and GES-1 cells compared to non-infected cells at 24 h after *H. pylori* infection. However, transfection of mir-30d mimic significantly decreased autophagic vacuoles in both cell types ($P < 0.05$, mimics *vs* control; Figure 2D).

Transfection of mir-30d inhibitor in AGS and GES-1 cell lines upregulates autophagy after H. pylori *infection*

For loss of function experiments, endogenous mir-30d expression was blocked by mir-30d inhibitor in both cell lines and then the cells were infected with *H. pylori*. The effects of blocked mir-30d expression on autophagy in both cells lines were examined by western blotting, GFP-LC3 puncta assay, and TEM at 24 h after *H. pylori* infection.

In Figure 3A, the expression of mir-30d was decreased obviously in AGS cell lines with or without *H. pylori* infection at 48 h after mir-30d inhibitor transfection ($P < 0.05$, *vs* control oligos transfected cells). A similar result was found in GES-1 cells ($P < 0.05$, oligos transfected cells *vs* control cells). Western blotting showed that autophagy was enhanced (increased LC3B-Ⅱ expression) in both cell lines during *H. pylori* infection. After transfection of mir-30d inhibitor into both cell lines, this process was further increased significantly (increased LC3B-Ⅱ conversion; Figure 3B).

Co-transfection of GFP-LC3 plasmid and mir-30d inhibitor/control oligos into AGS and GES-1 cells was done using the lipofectamine RNAi MAX transfection reagent for 24 h and then the cells were infected with *H. pylori*. Cells were imaged under confocal laser-scanning microscope 24 h later. The results showed

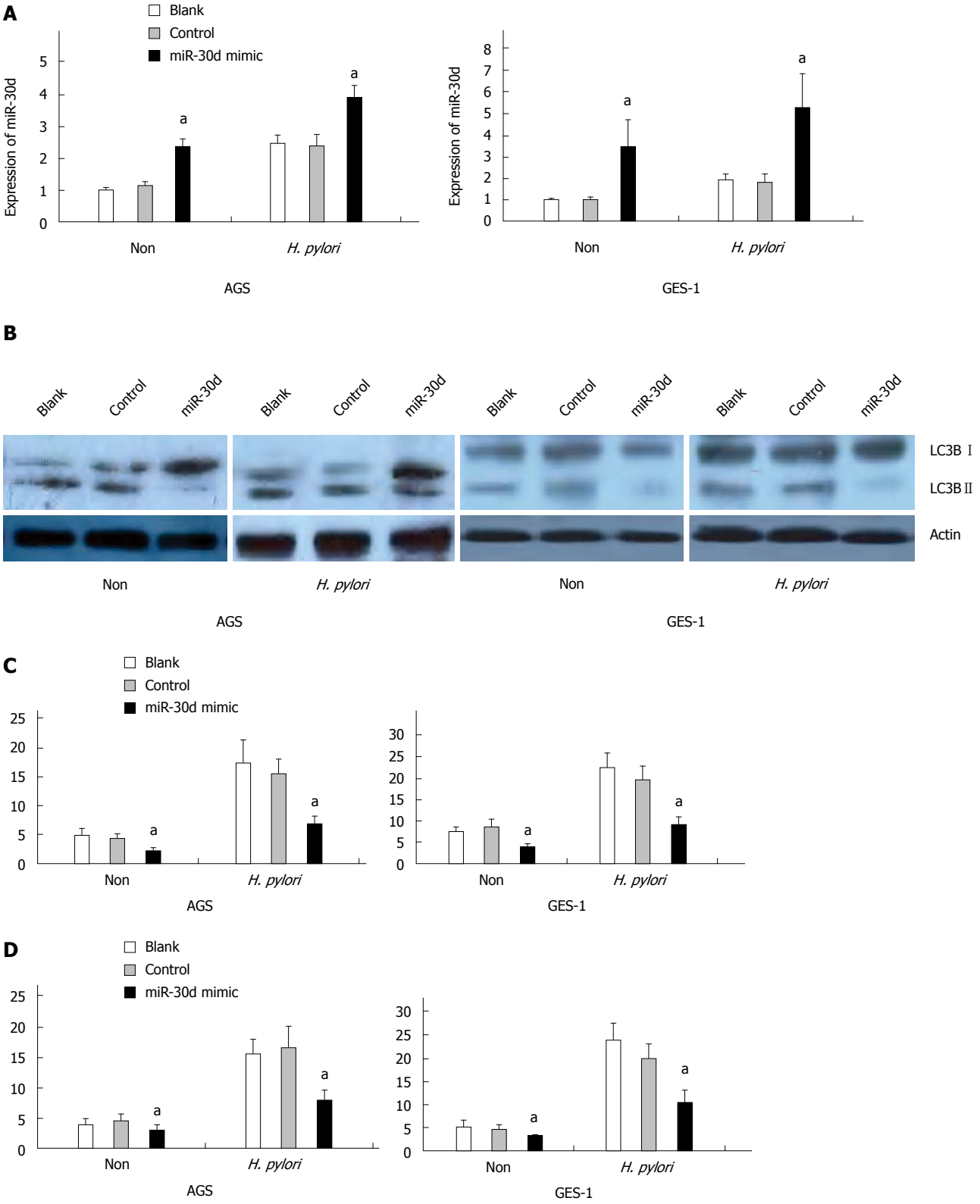


Figure 2 Mir-30d mimic represses autophagy in response to *Helicobacter pylori* infection in AGS and GES-1 cell lines. A: mir-30d expression in AGS and GES-1 cells with or without *H. pylori* infection at 48 h after mir-30d mimic transfection. Results shown as mean ± SD, $^{a}P < 0.05$ *vs* control; B: The protein levels of LC3B-I and LC3B-Ⅱ in mir-30d mimic transfected AGS and GES-1 cells with or without 24 h *H. pylori* infection; C: GFP-LC3 puncta in mir-30d transfected AGS and GES-1 cells with or without *H. pylori* infection (Results shown as mean ± SD, $^{a}P < 0.05$ *vs* control mimic transfected cells); D: Quantification of autophagosome and autophagolysosome in mir-30d mimic transfected AGS and GES-1 cells with or without 24 h *H. pylori* infection (results shown as mean ± SD, $^{a}P < 0.05$ *vs* control mimic transfected cells). *H. pylori*: *Helicobacter pylori*.

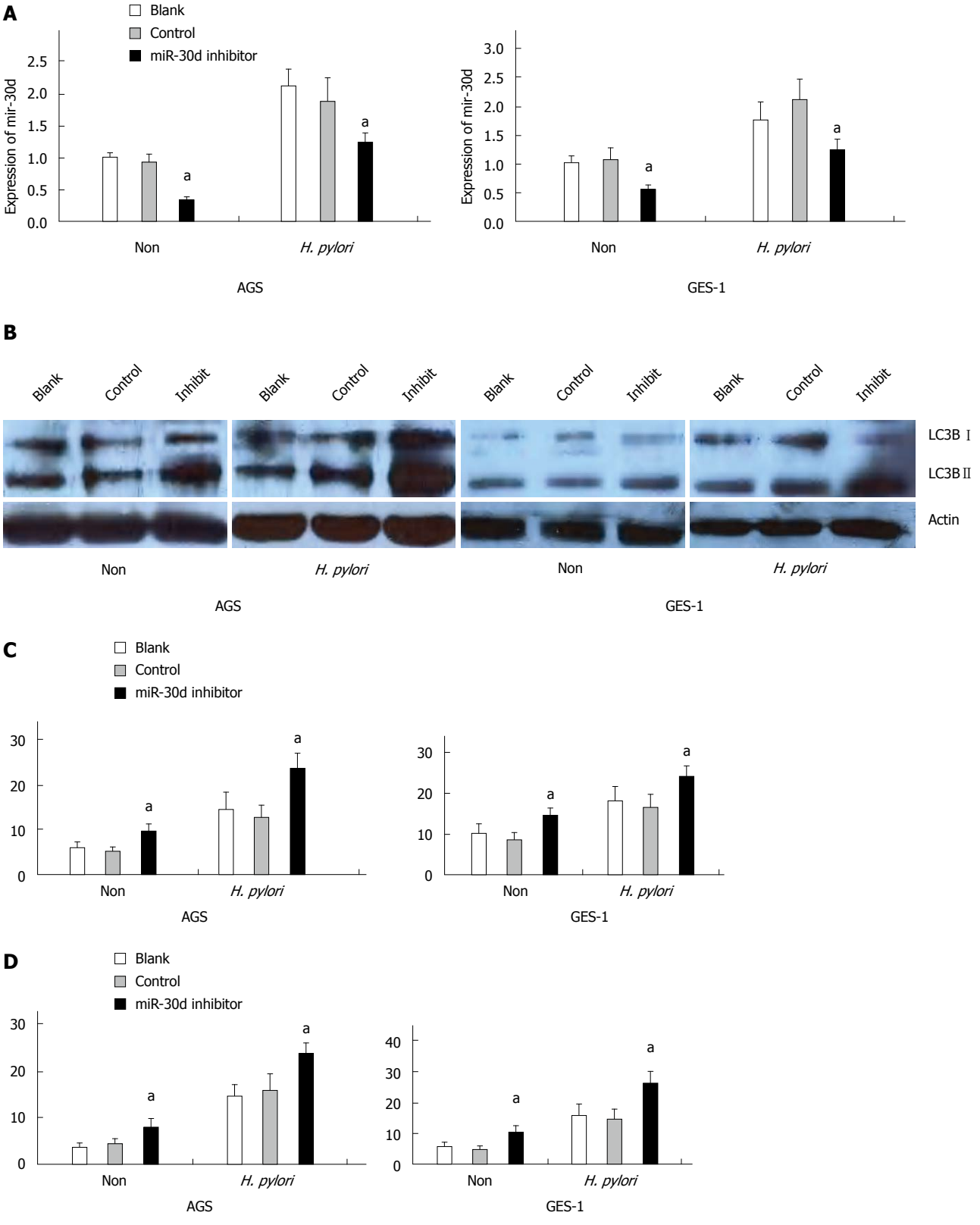


Figure 3 Mir-30d inhibitor upregulates autophagy in response to *Helicobacter pylori* infection in AGS and GES-1 cell lines. A: mir-30d expression in AGS and GES-1 cells with or without *H. pylori* infection at 48 h after mir-30d inhibitor transfection. $^{a}P < 0.05$, with mir-30d inhibitor *vs* without mir-30d inhibitor; B: LC3B-I and LC3B-Ⅱ protein levels in AGS and GES-1 cells with or without 24 h *H. pylori* infection; C: GFP-LC3 puncta in mir-30d transfected AGS and GES-1 cells with or without *H. pylori* infection (results shown as mean ± SD, $^{a}P < 0.05$ *vs* control oligos transfected cells); D: Autophagosome and autophagolysosome in mir-30d transfected AGS and GES-1 cells with or without *H. pylori* infection (results shown as mean ± SD, $^{a}P < 0.05$ *vs* control oligos transfected cells). *H. pylori*: *Helicobacter pylori*.

that GFP-LC3 puncta significantly increased in AGS and GES-1 cells infected with *H. pylori* as compared with non-infected cells. Nevertheless, mir-30d inhibitor significantly increased GFP-LC3 positive puncta in both cell types ($P < 0.05$, oligos transfected cells *vs* control cells, Figure 3C).

TEM assay also showed that there was an increased number of autophagic vacuoles (AV) in *H. pylori* infected AGS and GES-1 cells in contrast to non-infected cells at 24 h after *H. pylori* infection. After transfection of mir-30d inhibitor into the above cells, autophagic vacuoles were increased in both cell types ($P < 0.05$, oligos transfected cells *vs* control; Figure 3D).

Mir-30d suppresses the expression of multiple core autophagy proteins in gastric epithelial cells

Previously, it was found that mir-30d inhibited the autophagy process in ovarian cancer and breast cancer cell lines by directly targeting multiple genes of the autophagy pathway, including BECN1, BNIP3L, ATG12, ATG5 and ATG2[25]. To test the effect of mir-30d inhibition on gastric epithelial cells infected with *H. pylori*, we prepared reporter plasmids containing wild type or mutant mir-30d binding sites from 3′ untranslated region (UTR) of target genes (ATG2B, ATG5, ATG12, BECN1 and BNIP3L) for luciferase activity assay. Co-transfection of luciferase reporter plasmids and mir-30d mimic or control oligos showed that mir-30d potently decreased the luciferase activity of wild type reporter plasmids that represented all five target genes examined (ATG2B, ATG5, ATG12, BECN1, and BNIP3L), whereas it had no effect on the mutant reporter plasmids (Figure 4A). Perhaps mir-30d suppressed autophagy pathway gene expression by binding to its binding site within the 3′UTR of the target genes in a sequence-specific manner.

To further validate the repression of mir-30d on targeted autophagic genes in the autophagy pathway, mir-30d mimics were transfected in AGS and GES-1 cells, and target gene expression was analyzed with qRT-PCR. The mRNA levels of ATG2B, ATG5, ATG12, BECN1, and BNIP3L were remarkably suppressed by mir-30d mimic transfection compared with mimic control transfected in both AGS and GES-1 cells (Figure 4B). Similar results were obtained using western blots to detect the protein levels of these mir-30d potential targets in the above mir-30d mimic or control mimic treated cells. The protein levels for ATG2B, ATG5, ATG12, BECN1, and BNIP3L were reduced by mir-30d mimic transfection (Figure 4C). These results suggested that mir-30d regulated these autophagic genes at both the mRNA and protein level.

A loss of function experiment was applied with the mir-30d inhibitor. When endogenous mir-30d expression was blocked in the above cell lines (AGS and GES-1), the mRNA levels of ATG2B, ATG5, ATG12, BECN1, and BNIP3L were detected through qRT-PCR. In both cell lines (Figure 4D), the mRNA levels of the above genes were significantly increased by mir-30d inhibitor compared to control oligos.

Mir-30d increases intracellular survival of H. pylori *in AGS cells through inhibition of autophagy*

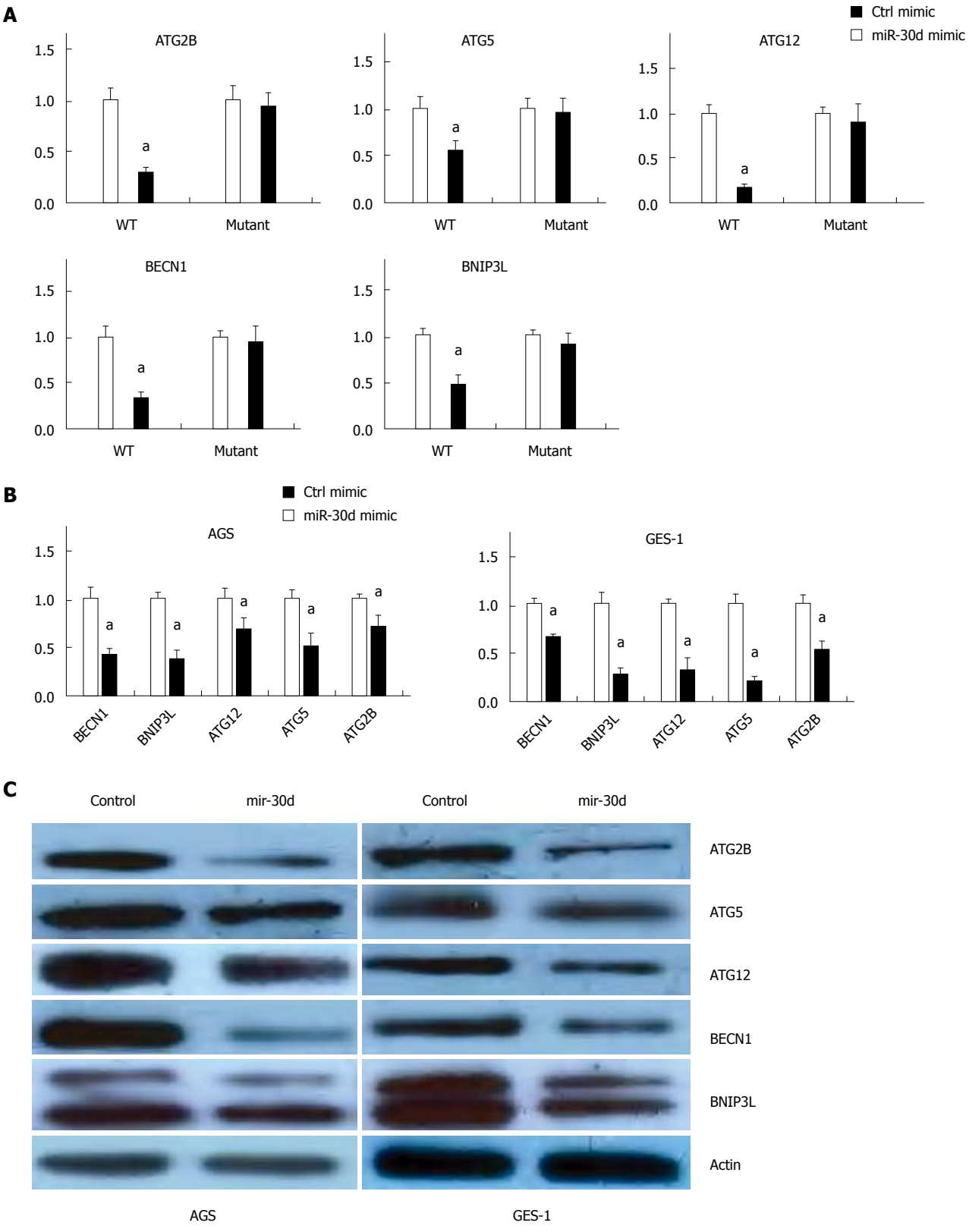
H. pylori invasion of gastric epithelial cells has been reported previously[24,26-28]. To evaluate the number of live internalized *H. pylori* cells, AGS cells were pretreated with PBS (as control), DMSO, autophagy inhibitor (3-MA), autophagy activators (starvation or rapamycin), mir-30d mimic, and mir-30d inhibitor, respectively, and then, a gentamicin protection assay was performed. The number of *H. pylori* CFU was increased approximately 10-fold 24 h after infection compared with 3 h of infection in all examined groups, indicating that internalized *H. pylori* underwent replication. Subsequently, the number of CFU decreased after 24 h in all groups (Figure 5A). However, compared with control (PBS) and DMSO groups, the number of CFU was obviously higher in mir-30d mimic and autophagy inhibitor 3-MA groups and lower in mir-30d inhibitor and autophagy activator (starvation or rapamycin) groups at all time points during a 60 h experiment (Figure 5A). The results from different treatments 24 h after infection with *H. pylori* are plotted in Figure 5B. These findings suggest that inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

DISCUSSION

H. pylori is a common phenomenon worldwide, reaching nearly one-half of the world's population. Chronic *H. pylori* infection is etiologically linked to gastric adenocarcinoma, especially non-cardia type (63% of all stomach cancer or 25% of cancers are associated with infectious etiology)[29]. These discoveries highlight the importance of basic research and clinical research on *H. pylori* infection and treatment. As resistance to the current proton pump inhibitor-based triple regimens or second-line therapies for the eradication of *H. pylori* continue to grow, so will the need to search for novel approaches.

It is known that varied autophagy is related to persistent *H. pylori* infection. Chu and colleagues[6] found that rapamycin, an inducer of autophagy, increased the clearance of *H. pylori*. However, they also found that many coccoid forms of *H. pylori* occurred on the membrane of the infected AGS cells. Autophagic vesicles were induced and their maturation was arrested with rapamycin[7], but it was not clear if *H. pylori* strains were killed inside the autophagic vesicles, as Amano *et al*[30] had indicated previously.

The role of autophagy in cancer development is important. Autophagy may be tumor-suppressing


A
Ctrl mimic
miR-30d mimic
ATG2B
ATG5
ATG12
BECN1
BNIP3L
1.5
1.0
0.5
0.0
a
WT
Mutant
B
Ctrl mimic
miR-30d mimic
AGS
GES-1
BECN1
BNIP3L
ATG12
ATG5
ATG2B
C
Control
mir-30d
Control
mir-30d
ATG2B
ATG5
ATG12
BECN1
BNIP3L
Actin
AGS
GES-1

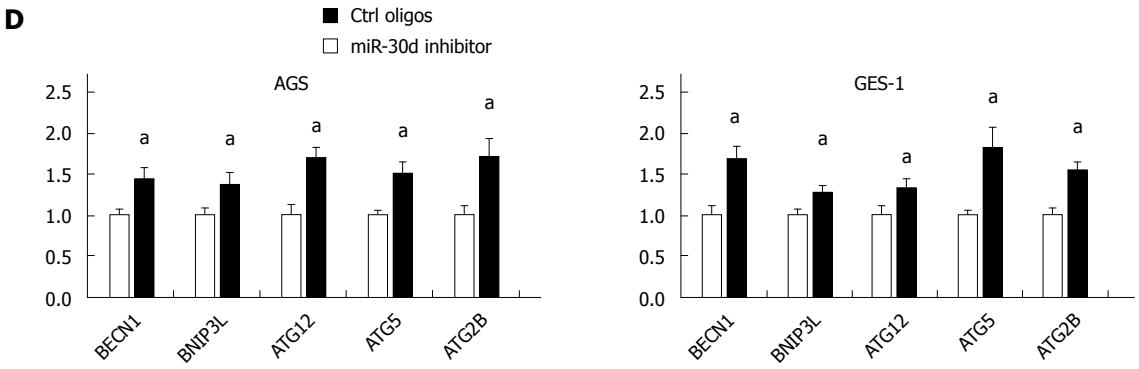


Figure 4 Multiple core proteins in the autophagy pathway are direct targets of mir-30d in gastric epithelial cells. A: Luciferase reporter assay with plasmids bearing wild type or mutant 3'UTR binding sites of mir-30d in AGS cells with mir-30d mimic or control oligos. Luciferase activity of mir-30d mimic transfected cells was normalized to control mimic transfected cells. Results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control; B: mRNA levels of autophagy related (ATG)2B, ATG5, ATG12, beclin 1 (BECN1), and BNip3-like protein (BNIP3L) in both cell lines with mir-30d mimic or control mimic transfection. Results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control; C: ATG2B, ATG5, ATG12, BECN1, and BNIP3L protein levels in both cell lines with mir-30d mimic or control mimic transfection; D: The mRNA levels of ATG2B, ATG5, ATG12, BECN1, and BNIP3L in two cell lines with or without mir-30d inhibitor transfection. Results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control.

during the early stages of tumorigenesis, as reduced expression of autophagy proteins was shown to contribute to the development or progression of human breast and other cancers[31-33]. Sometimes, however, autophagy promotes cancer development[25]. In this case, downregulation of autophagy may benefit the intracellular survival of *H. pylori*, and induction of autophagy may be beneficial indirectly for cancer prevention.

Recently, miRNAs were demonstrated to play a crucial role in autophagy regulation, such as mir-30a, mir-30b, mir-17/20/93/106, mir-204, and mir-10b[15,26,34-36]. Tang *et al*[15] found that compromised autophagy by mir-30b led to a failure to clear intracellular *H. pylori*, resulting in persistent *H. pylori* infection and proliferation in the host cells. Kobayashi *et al*[37] suggested that mir-30d is a prognostic maker for prostate cancer. Our previous study showed that mir-30d regulated the autophagy process by directly targeting multiple autophagic genes in the autophagy pathway[25].

In this study, we confirmed a hypothesis that mir-30d could inhibit autophagy in gastric epithelial cells induced by *H. pylori* invasion by down-regulating autophagy-related gene expression, resulting in increased *H. pylori* intracellular survival. The results obtained from GFP-LC3 puncta assay, TEM, and western blot demonstrated that autophagy could be induced in AGS and GES-1 cells in response to *H. pylori* infection. The expression of mir-30d was upregulated in both cells after *H. pylori* infection in our experiments. This event appeared to be unique to *H. pylori* infection, but it must be repeated with other pathogens to demonstrate its specificity. Study on mir-30b found that infection with other pathogens (*E. coli* DH5α and O157:H7) or autophagy modulators (*e.g.*, rapamycin and 3-methyladenine) had no effect on mir-30b expression[15]. We found that autophagy was upregulated in both cell lines after *H. pylori* infection, but upregulation of mir-30d significantly inhibited this process. In contrast, when mir-30d expression was blocked by mir-30d inhibitor, autophagy was obviously increased by downregulation of mir-30d. Mir-30d also repressed the autophagy process by directly targeting multiple core genes (ATG2B, ATG5, ATG12, BECN1, and BNIP3L). A gentamicin protection assay indicated that inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

Although overexpression of mir-30d could decrease autophagy by inhibiting the expression of multiple core genes of the autophagy pathway in gastric epithelial cells, the regulation event might have happened after *H. pylori*-induced autophagy. Moreover, downregulation of autophagy by mir-30d may not be sufficient to block the autophagy induced by *H. pylori*. In assayed AGS and GES-1 cells with exogenously added mir-30d mimic, autophagy in *H. pylori* infection was more than that in uninfected cells (Figure 2B, C and D). Given the complexity of *H. pylori* infection *in vivo*, other factors may also contribute to autophagy inhibition. As one of the factors, overexpression of mir-30d may slightly continue to inhibit autophagy pathway for a long time, leading to subversion of host autophagic responses for their survival or growth.

Based on the above results, we concluded that repression of autophagy by mir-30d may help intracellular *H. pylori* evade autophagic clearance through targeting ATG2B, ATG5, ATG12, BECN1, and BNIP3L. These findings provide a novel molecular mechanism for persistent *H. pylori* occupancy. Although much remains to be studied on the regulation of autophagy in gastric cancer, the current study provides a promising target for gastric cancer prevention. We suggest that enhanced autophagy by mir-30d inhibition may be protective against *H. pylori*-related gastric cancer.

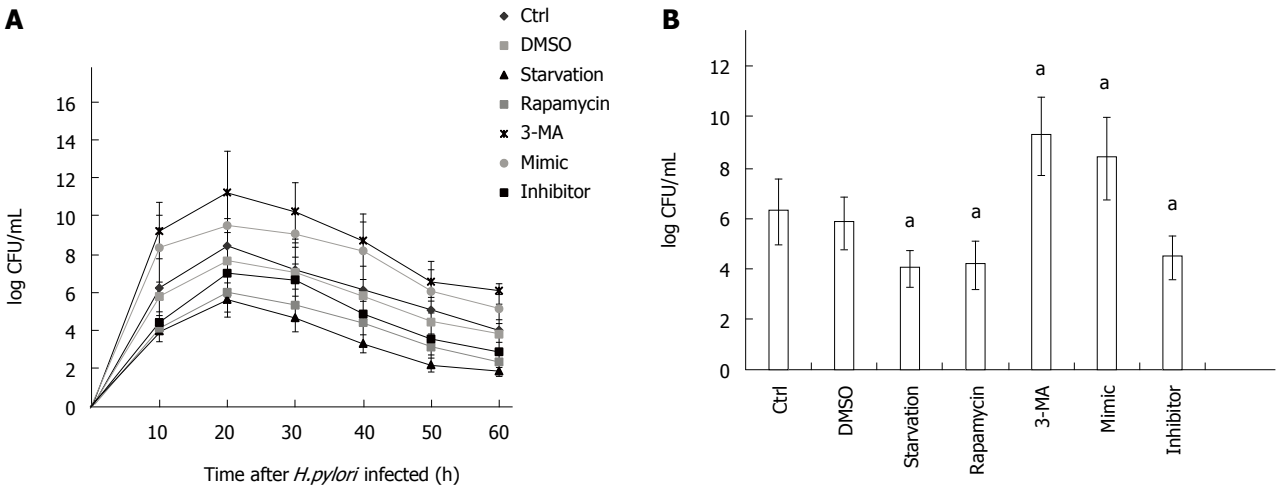


Figure 5 Mir-30d increases intracellular survival of *Helicobacter pylori* in AGS cells. A: Gentamicin protection assay for the number of colony forming units (CFU) of *H. pylori* during a 60 h *H. pylori* infection with different treatment; B: The results from 24 h *H. pylori* infection, results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control. *H. pylori*: *Helicobacter pylori*.

Preventive measures for gastric cancer must include tertiary prevention and effective treatment of *H. pylori* infections. The long-term decline in gastric cancer mortality in developed countries has resulted, in part, from interrupting *H. pylori* transmission through provision of improved basic sanitation, housing, and socioeconomic status. However, secondary prevention may be attempted where simple diagnostic tests, follow-up treatment (urea breath test), and effective, short-term eradication treatment are available to mitigate individual risk[38].

In summary, our report indicates a novel molecular mechanism for the inhibition of autophagy by mir-30d by increasing the intracellular survival of *H. pylori*. Although a detailed mechanism for *H. pylori* persistence remains to be elucidated, the present study establishes a basis that will be helpful for future evaluation of mir-30d in *H. pylori* infections.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) was designated as a class I carcinogen by the International Agency for Research on Cancer in 1994 due to its strong correlation with gastric cancer in humans. One possible hypothesis for the relatively high resistance to therapy may be the ability of *H. pylori* to reside inside host cells.

Research frontiers

Over the last decade, several research groups have independently reported that infection by *H. pylori* can induce macroautophagy. However, *H. pylori* has been reported to evade the autophagic machinery by downregulating the expression of autophagic proteins.

Innovations and breakthroughs

In this study, the authors confirmed that mir-30d could represses autophagy in response to *H. pylori* invasion by directly targeting multiple core genes of the autophagy pathway in gastric epithelial cells, including *ATG2B*, *ATG5*, *ATG12*, *BECN1* and *BNIP3L*. Inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

Applications

These findings may provide a novel mechanism for elucidating persistent *H. pylori* infection, and it appears to provide a promising target for gastric cancer prevention. Although the mechanism of *H. pylori* infection persistence remains to be fully determined, the present study provides the basis for future evaluations of mir-30d in *H. pylori* infections.

Terminology

Autophagy, which is present in cells at a low level basally, is an evolutionarily conserved process for delivering cellular materials and organelles to lysosome for degradation within double-membraned vacuoles, called autophagosomes. Autophagy is also regarded as one of the innate immunity effectors against intracellular bacterial infection.

Peer-review

This is a very good study on mir-30d and *H. pylori* in gastric epithelial cells. Mir-30d was shown to inhibit multiple core proteins in the autophagy pathway. The link between mir-30d in gastric cancer and *H. pylori* is novel, as no other study has indicated previously this relationship in the literature.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; **94**: 153-156 [PMID: 11668491 DOI: 10.1002/ijc.1440]
2 **Hartgrink HH**, Jansen EP, van Grieken NC, van de Velde CJ. Gastric cancer. *Lancet* 2009; **374**: 477-490 [PMID: 19625077 DOI: 10.1016/S0140-6736(09)]
3 **Vogiatzi P**, Cassone M, Luzzi I, Lucchetti C, Otvos L, Giordano A. Helicobacter pylori as a class I carcinogen: physiopathology and management strategies. *J Cell Biochem* 2007; **102**: 264-273 [PMID: 17486575 DOI: 10.1002/jcb.21375]
4 **Wang YH**, Wu JJ, Lei HY. The autophagic induction in Helicobacter pylori-infected macrophage. *Exp Biol Med* (Maywood) 2009; **234**: 171-180 [PMID: 19064937 DOI: 10.3181/0808-RM-252]
5 **Allen LA**. Phagocytosis and persistence of Helicobacter pylori. *Cell Microbiol* 2007; **9**: 817-828 [PMID: 17346311 DOI: 10.1111/j.1462-5822.2007.00906.x]
6 **Chu YT**, Wang YH, Wu JJ, Lei HY. Invasion and multiplication of Helicobacter pylori in gastric epithelial cells and implications for antibiotic resistance. *Infect Immun* 2010; **78**: 4157-4165 [PMID: 20696835 DOI: 10.1128/IAI.00524-10]
7 **Rabinowitz JD**, White E. Autophagy and metabolism. *Science* 2010; **330**: 1344-1348 [PMID: 21127245 DOI: 10.1126/science.1193497]

8 **Levine B**, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008; **132**: 27-42 [PMID: 18191218 DOI: 10.1016/j.cell.2007.12.018]

9 **Yoshimori T**, Amano A. Group a Streptococcus: a loser in the battle with autophagy. *Curr Top Microbiol Immunol* 2009; **335**: 217-226 [PMID: 19802567 DOI: 10.1007/978-3-642-00302-8_10]

10 **Nakagawa I**, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, Nara A, Funao J, Nakata M, Tsuda K, Hamada S, Yoshimori T. Autophagy defends cells against invading group A Streptococcus. *Science* 2004; **306**: 1037-1040 [PMID: 15528445 DOI: 10.1126/science.1103966]

11 **Ogawa M**, Sasakawa C. Shigella and autophagy. *Autophagy* 2006; **2**: 171-174 [PMID: 16874102 DOI: 10.4161/auto.2829]

12 **Ogawa M**, Mimuro H, Yoshikawa Y, Ashida H, Sasakawa C. Manipulation of autophagy by bacteria for their own benefit. *Microbiol Immunol* 2011; **55**: 459-471 [PMID: 21707736 DOI: 10.1111/j.1348-0421.2011.00343.x]

13 **Wang YH**, Gorvel JP, Chu YT, Wu JJ, Lei HY. Helicobacter pylori impairs murine dendritic cell responses to infection. *PLoS One* 2010; **5**: e10844 [PMID: 20523725 DOI: 10.1371/journal.pone.0010844]

14 **Terebiznik MR**, Raju D, Vázquez CL, Torbricki K, Kulkarni R, Blanke SR, Yoshimori T, Colombo MI, Jones NL. Effect of Helicobacter pylori's vacuolating cytotoxin on the autophagy pathway in gastric epithelial cells. *Autophagy* 2009; **5**: 370-379 [PMID: 19164948 DOI: 10.4161/auto.5.3.7663]

15 **Tang B**, Li N, Gu J, Zhuang Y, Li Q, Wang HG, Fang Y, Yu B, Zhang JY, Xie QH, Chen L, Jiang XJ, Xiao B, Zou QM, Mao XH. Compromised autophagy by MIR30B benefits the intracellular survival of Helicobacter pylori. *Autophagy* 2012; **8**: 1045-1057 [PMID: 22647547 DOI: 10.4161/auto.20159]

16 **Bar M**, Wyman SK, Fritz BR, Qi J, Garg KS, Parkin RK, Kroh EM, Bendoraite A, Mitchell PS, Nelson AM, Ruzzo WL, Ware C, Radich JP, Gentleman R, Ruohola-Baker H, Tewari M. MicroRNA discovery and profiling in human embryonic stem cells by deep sequencing of small RNA libraries. *Stem Cells* 2008; **26**: 2496-2505 [PMID: 18583537 DOI: 10.1634/stemcells.2008-0356]

17 **Stark MS**, Tyagi S, Nancarrow DJ, Boyle GM, Cook AL, Whiteman DC, Parsons PG, Schmidt C, Sturm RA, Hayward NK. Characterization of the Melanoma miRNAome by Deep Sequencing. *PLoS One* 2010; **5**: e9685 [PMID: 20300190 DOI: 10.1371/journal.pone.0009685]

18 **Bridge G**, Monteiro R, Henderson S, Emuss V, Lagos D, Georgopoulou D, Patient R, Boshoff C. The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis. *Blood* 2012; **120**: 5063-5072 [PMID: 23086751 DOI: 10.1182/blood-2012-04-423004]

19 **Franzetti GA**, Laud-Duval K, Bellanger D, Stern MH, Sastre-Garau X, Delattre O. MiR-30a-5p connects EWS-FLI1 and CD99, two major therapeutic targets in Ewing tumor. *Oncogene* 2013; **32**: 3915-3921 [PMID: 22986530 DOI: 10.1038/onc.2012.403]

20 **Quintavalle C**, Donnarumma E, Iaboni M, Roscigno G, Garofalo M, Romano G, Fiore D, De Marinis P, Croce CM, Condorelli G. Effect of miR-21 and miR-30b/c on TRAIL-induced apoptosis in glioma cells. *Oncogene* 2013; **32**: 4001-4008 [PMID: 22964638 DOI: 10.1038/onc.2012.410]

21 **Martinez I**, Cazalla D, Almstead LL, Steitz JA, DiMaio D. miR-29 and miR-30 regulate B-Myb expression during cellular senescence. *Proc Natl Acad Sci USA* 2011; **108**: 522-527 [PMID: 21187425 DOI: 10.1073/pnas.1017346108]

22 **Baraniskin A**, Birkenkamp-Demtroder K, Maghnouj A, Zöllner H, Munding J, Klein-Scory S, Reinacher-Schick A, Schwarte-Waldhoff I, Schmiegel W, Hahn SA. MiR-30a-5p suppresses tumor growth in colon carcinoma by targeting DTL. *Carcinogenesis* 2012; **33**: 732-739 [PMID: 22287560 DOI: 10.1093/carcin/bgs020]

23 **Li N**, Kaur S, Greshock J, Lassus H, Zhong X, Wang Y, Leminen A, Shao Z, Hu X, Liang S, Katsaros D, Huang Q, Bützow R, Weber BL, Coukos G, Zhang L. A combined array-based comparative genomic hybridization and functional library screening approach identifies mir-30d as an oncomir in cancer. *Cancer Res* 2012; **72**: 154-164 [PMID: 22058146 DOI: 10.1158/0008-5472.CAN-11-2484]

24 **Lu Y**, Ryan SL, Elliott DJ, Bignell GR, Futreal PA, Ellison DW, Bailey S, Clifford SC. Amplification and overexpression of Hsa-miR-30b, Hsa-miR-30d and KHDRBS3 at 8q24.22-q24.23 in medulloblastoma. *PLoS One* 2009; **4**: e6159 [PMID: 19584924 DOI: 10.1371/journal.pone.0006159]

25 **Yang X**, Zhong X, Tanyi JL, Shen J, Xu C, Gao P, Zheng TM, DeMichele A, Zhang L. mir-30d Regulates multiple genes in the autophagy pathway and impairs autophagy process in human cancer cells. *Biochem Biophys Res Commun* 2013; **431**: 617-622 [PMID: 23274497 DOI: 10.1016/j.bbrc.2012.12.083]

26 **Zhu H**, Wu H, Liu X, Li B, Chen Y, Ren X, Liu CG, Yang JM. Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. *Autophagy* 2009; **5**: 816-823 [PMID: 19535919 DOI: 10.4161/auto.9064]

27 **Zou Z**, Wu L, Ding H, Wang Y, Zhang Y, Chen X, Chen X, Zhang CY, Zhang Q, Zen K. MicroRNA-30a sensitizes tumor cells to cis-platinum via suppressing beclin 1-mediated autophagy. *J Biol Chem* 2012; **287**: 4148-4156 [PMID: 22157765 DOI: 10.1074/jbc.M111.307405]

28 **Xiao B**, Li W, Guo G, Li B, Liu Z, Jia K, Guo Y, Mao X, Zou Q. Identification of small noncoding RNAs in Helicobacter pylori by a bioinformatics-based approach. *Curr Microbiol* 2009; **58**: 258-263 [PMID: 19123032 DOI: 10.1007/s00284-008-9318-2]

29 **Parkin DM**. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006; **118**: 3030-3044 [PMID: 16404738 DOI: 10.1002/ijc.21731]

30 **Amano A**, Nakagawa I, Yoshimori T. Autophagy in innate immunity against intracellular bacteria. *J Biochem* 2006; **140**: 161-166 [PMID: 16954534]

31 **Liang XH**, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999; **402**: 672-676 [PMID: 10604474 DOI: 10.1038/45257]

32 **Takamura A**, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K, Mizushima N. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* 2011; **25**: 795-800 [PMID: 21498569 DOI: 10.1101/gad.2016211]

33 **Yue Z**, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci USA* 2003; **100**: 15077-15082 [PMID: 14657337 DOI: 10.1073/pnas.2436255100]

34 **Meenhuis A**, van Veelen PA, de Looper H, van Boxtel N, van den Berge IJ, Sun SM, Taskesen E, Stern P, de Ru AH, van Adrichem AJ, Demmers J, Jongen-Lavrencic M, Löwenberg B, Touw IP, Sharp PA, Erkeland SJ. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood* 2011; **118**: 916-925 [PMID: 21628417 DOI: 10.1182/blood-2011-02-336487]

35 **Xiao J**, Zhu X, He B, Zhang Y, Kang B, Wang Z, Ni X. MiR-204 regulates cardiomyocyte autophagy induced by ischemia-reperfusion through LC3-II. *J Biomed Sci* 2011; **18**: 35 [PMID: 21631941 DOI: 10.1186/1423-0127-18-35]

36 **Gabriely G**, Teplyuk NM, Krichevsky AM. Context effect: microRNA-10b in cancer cell proliferation, spread and death. *Autophagy* 2011; **7**: 1384-1386 [PMID: 21795860 DOI: 10.4161/auto.7.11.17371]

37 **Kobayashi N**, Uemura H, Nagahama K, Okudela K, Furuya M, Ino Y, Ito Y, Hirano H, Inayama Y, Aoki I, Nagashima Y, Kubota Y, Ishiguro H. Identification of miR-30d as a novel prognostic maker of prostate cancer. *Oncotarget* 2012; **3**: 1455-1471 [PMID: 23231923]

38 **Leung WK**, Wu MS, Kakugawa Y, Kim JJ, Yeoh KG, Goh KL,

Wu KC, Wu DC, Sollano J, Kachintorn U, Gotoda T, Lin JT, You WC, Ng EK, Sung JJ. Screening for gastric cancer in Asia: current evidence and practice. *Lancet Oncol* 2008; **9**: 279-287 [PMID: 18308253 DOI: 10.1016/S1470-2045(08)70072-X]

P- Reviewer: Kocazeybek B, Romo-Gonzalez C
S- Editor: Qi Y **L- Editor**: Filipodia **E- Editor**: Liu XM

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 1007-9327
15>