Original Article

MicroRNA-155-enhanced Autophagy in Human Gastric Epithelial Cell in Response to *Helicobacter Pylori*

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ABSTRACT

Background/Aim: MicroRNAs (miRNAs) are a class of small noncoding RNAs acting as posttranscriptional gene expression regulators in many physiological and pathological conditions. MiR-155 is one kind of miRNAs that plays an important role in causing various diseases. However, the precise molecular mechanism of the ectopic expression of miR-155 in *Helicobacter pylori* infection remains poorly understood. Autophagy has recently been identified as an effective way to control the intracellular bacterium survival. In the present study, we demonstrate a novel role of miR-155 in regulating the autophagy-mediated anti-*H. pylori* response. **Patients and Methods:** Totally 86 *H. pylori*-positive patients together with 10 *H. pylori*-negative, healthy control subjects were included in the study. Correlation between immunohistochemical grades and miR-155 expression were determined. Molecular mechanism of miR-155 on regulation of autophagy and elimination of intracellular *H. pylori* were determined using the GES-1 cell model. **Results:** We found that overexpression of miR-155 by transfecting miR-155 mimics could significantly decrease the survival of intracellular *H. pylori*, and this process was through induction of autophagy. Furthermore, there was a significant correlation between miR-155 and immunohistochemical grades in *H. pylori*-positive patients, and miR-155 expression were decreased in the intestinal metaplasia group. **Conclusions:** The results have indicated that the miR-155 expression level plays a key role in immunity response against *H. pylori* and this might provide potential targets for the future treatment of *H. pylori*-related diseases.

Key Words: Autophagy, *Helicobacter pylori*, intestinal metaplasia, microRNA

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Helicobacter pylori is a gram-negative bacterium that plays an etiologic role in gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer.[1] Although *H. pylori* is generally considered as an extracellular microorganism, a growing number of evidence supports that at least a subset of *H. pylori* has an intraepithelial location and a minor fraction of *H. pylori* resides inside gastric epithelial cells. *H. pylori* is capable of surviving and persisting in host cells because of its repertoire of evading the host immune response.[2] Meanwhile, host cells also deploy a multitude of immune defense mechanisms to kill this pathogen. Scientists believe that a better understanding of the complex network within *H. pylori*-infected host surroundings might help to explore the diagnostic and treatment method.

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Autophagy is an evolutionarily conserved process, which is involved in maintaining cytoplasmic homeostasis by degrading damaged organelles or misfolded proteins.[3] The autophagic cascade is initiated by the engulfment of cytoplasmic cargoes by an autophagosome, which then fuses with a late endosome to form the autolysosome, exposing the inner compartment to lysosomal hydrolases for degradation.[4] Recently, studies have suggested that autophagy may link the multistep process of intracellular *H. pylori* clearance.[5] Additionally, *H. pylori* can induce autophagy in gastric epithelial cells.[6] However, the mechanism by which *H. pylori* antagonize host autophagy remains to be elucidated.

As a class of small noncoding RNAs, miRNAs are highly conserved between different eukaryotic species and they function as key regulators of gene expression at the post-transcriptional level by targeting mRNAs for translational repression or degradation. Previous work has

reported that microRNA-155 negatively regulated the release of proinflammatory cytokines and signal transduction during *H. pylori* infection.[7] In this study, we aim to describe our new discovery about the regulatory role of microRNA-155 in autophagy triggered by *H. pylori* infection in gastric epithelial cell.

PATIENTS AND METHODS

Antibodies and reagents

The GFP-LC3B plasmid was provided by Dr. Liwei Dong (International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai); the pcDNA-3.1-Rheb plasmid and pcDNA-3.1-3- RPS6KB2 plasmid were obtained from RiboBio Co., Ltd, Guangzhou; methyladenine (3-MA, M9281) and Rapamycin (Rapa, R8781) were purchased from Sigma; Antibodies against LC3B (L7543) were obtained from Sigma; antibodies for Rheb (4935), RPTOR (2280), RICTOR (2114), RPS6KB2 (2708), and MTOR (2972) were obtained from cell signaling.

Cell and bacteria culture

The nonmalignant gastric epithelial cell lines GES-1 were routinely cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin in a humidified incubator containing 5% CO_2 at 37°C. The wild-type *H. pylori* strain 26695 was obtained from ATCC and grown as previously described.[8] Subsequently, cells were seeded to the wells of a 12-well plate and grown to 80% confluency. Then, the medium was replaced with antibiotic-free medium. *H. pylori* was added to cells at a multiplicity of infection of 100:1. The infection model was monitored by the release of interleukin-8 (IL-8) and growth-related oncogene–alpha (GRO-alpha), as measured by DuoSet ELISA Development System (R and D).

Cell transfection

All oligonucleotides were synthesized from GenePharma (Shanghai, P. R. China) products. Transfections were performed using Lipofectamine 2000 (Invitrogen). Cells were transfected with 50 nM miRNA mimics, inhibitors, or scrambled miR-control for 24 h.

Clinical samples

For clinical samples, a total number of 86 *H. pylori*-positive patients together with 10 *H. pylori*- negative but healthy control subjects were included in the study. The *H. pylori* infection status was confirmed by bacterial culture, C^{13}-urea breath test, and the histologic testing. Patients were regarded as being *H. pylori* positive if one of the tests yielded positive results. Patients with a history of gastric surgery, active gastrointestinal bleeding, use of steroids, immunosuppressive drugs, NSAIDs, proton pump inhibitors, or who were treated for *H. pylori* eradication were excluded from the study. Gastric fragments were obtained during endoscopy from five different sites as recommended by the Updated Sydney System for classification of gastritis.[9] A number of clinicopathological variables such as gender, age, tumor location, histological type, tumor-node-metastasis (TNM) stage, depth of tumor invasion, lymph node metastasis, distant metastasis, and vascular invasion were obtained from the histopathological records and included for survival analysis. The tissue sections were also assessed for the presence of *H. pylori* infection by immunohistochemical staining using polyclonal anti-*H. pylori* antibody. Presence of *H. pylori* was graded according to the following criteria: Grade 0 (0 bacteria/oil immersion field), Grade 1 (19 bacteria/oil immersion field), Grade 2 (20–29 bacteria/oil immersion field), Grade 3 (30–99 bacteria/oil immersion field), and Grade 4 (≥100 bacteria/oil immersion field).

Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from cells using the Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis for miRNAs was performed by using TaqMan miRNA assays (Ambion) in an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reverse transcription reactions were performed using the following parameters: 16°C, 30 min, 42°C, 30 min, and 84°C, 5 min. PCR reactions were performed using the following parameters: 95°C for 2 min followed by 40 cycles of 95°C, 15 s, and 60°C, 30 s. U6 small nuclear RNA was used as endogenous control for data normalization. Relative expression was calculated using the comparative threshold cycle (Ct) method. qRT-PCR analysis for the mRNAs of PTGS2 and β-actin was performed by using PrimeScript RT-PCR kits (Takara, Shiga, Japan). The mRNA level of β-actin was used as an internal control. The sequences of primers used were described in accordance with the previous report.[10]

Western blot

Cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed with protein lysate (Pierce, Rockford, IL, USA). After centrifugation at 5000 × g for 15 min at 4°C, the protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce). Fifty microgram aliquots of lysates were loaded on a sodium dodecylsulfate (SDS) polyacrylamide 10% gradient gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20, and were incubated with primary antibodies (1:200; Santa Cruz, CA, USA) and horseradish peroxidase-conjugated

secondary antibodies (1:5000; Santa Cruz) in accordance with manufacturer's instructions. The protein of interest was visualized using an enhanced chemiluminescence (ECL) Western blotting substrate (Pierce) and the Chemidoc XRS Gel Documentation System (BioRad).

Statistical analyses

The results are expressed as means ± SD from at least three separate experiments performed in triplicate. The difference between groups was determined using two-tailed Student's *t*-test by using SPSS software (Armonk, NY, USA). *P* values of less than 0.05 were considered significant. The Chi-square test or Fisher's exact test was used to analyze the relationship between miR-155 expression and the clinicopathological features.

RESULTS

miR-155 decreases the survival of intracellular H. pylori

GES-1 cells were transiently transfected with miR-155 mimic or inhibitor, and then challenged with *H. pylori* at an MOI of 100. Intracellular survival of *H. pylori* in GES-1 cells was examined at different times. Our results showed that miR-155 significantly reduced the survival of intracellular *H. pylori*, whereas transfection with miR-155 inhibitor returned the opposite results [Figure 1]. These results indicated that miR-155 could enhance the host killing ability against intracellular *H. pylori*.

miR-155 induces autophagy and promotes the elimination of intracellular H. pylori in GES-1 cell

Western-blot results showed that miR-155 enhanced the ratio of LC3-II/GAPDH, whereas miR-155 inhibitor reduced the LC3B-II/GAPDH ratio [Figure 2a]. To further confirm that miR-155 triggered the autophagy process, we used a GFP-LC3-II puncta formation assay to monitor autophagy. Transfection with miR-155 displayed a significant increase in the percentage of cells with autophagosomes (GFP-LC3-II dots) compared with mock-infected GES-1 cells ($P < 0.05$) [Figure 2b]. The collective data above demonstrated that miR-155 elevated the autophagic response in GES-1 cell.

We next examined whether miR-155 decreased the survival of intracellular *H. pylori* by induction of autophagy. GES-1 cells were pretreated with miR-155 mimic or inhibitor, and either left untreated or pretreated with 3-methyladenine (3-MA) or Rapamycin, followed by exposing to *H. pylori* for 24 h. We have found that Rapamicyin helped miR-155 to reduce the survival of intracellular *H. pylori*, whereas 3-MA blocked this reduction [Figure 3a]. As expected, the opposite results were seen in the miR-155 inhibitor group [Figure 3b]. These data demonstrated that miR-155 decreased the survival of intracellular *H. pylori* by inducing the autophagy process.

miR-155 induces autophagy by inhibiting Rheb

The previous report has demonstrated that miR-155 induced autophagy by targeting multiple players in the MTOR pathway.[10] Therefore, we then selected Rheb, RPTOR, RICTOR, RPS6KB2, and MTOR, which 3′- UTRs complementary to miR-155 were confirmed previously, to further examine the possible regulatory mechanism of microRNA-155-induced autophagy. First, we measured both the mRNA and the protein levels of these targets in GES-1 cells transfected with miR-155 mimics or inhibitors.

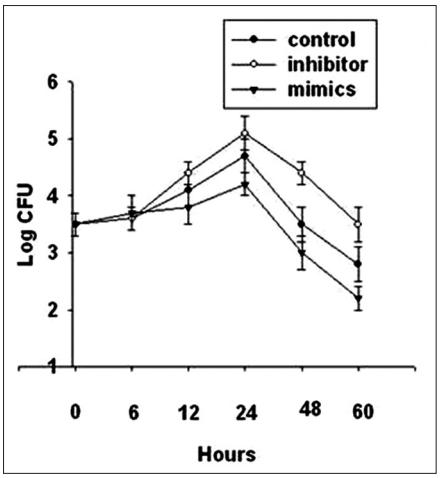

Figure 1: miR-155 decreases the survival of intracellular Helicobacter pylori in GES-1 cells. GES-1 cells were transiently transfected with negative control (NC), miR-155 mimic or miR-155 inhibitor, and then challenged with *H. pylori* at an MOI of 100, and intracellular *H. pylori* viability was determined by CFU counting at certain timepoint. MOI,: multiplicities of infection; CFU, colony forming units

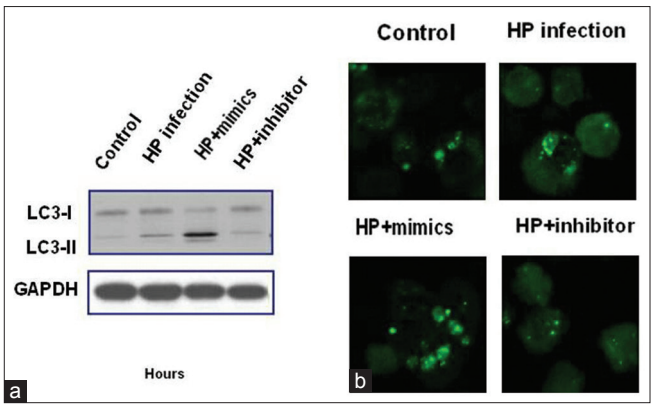

Figure 2: miR-155 induces autophagy in GES-1 cells. (a) GES-1 cells were transfected with negative control (NC), miR-155 mimic or miR-155 inhibitor for 24 h followed by *Helicobacter pylori* infection (MOI = 100:1), and the ratio of LC3B-II/GAPDH were determined. (b) GES-1 cells were transfected with plasmid expressing GFP-LC3B, together with negative control (NC), miR-155 mimics or inhibitor. After 24 h, the cells were incubated for 6 h at 37°C in F12 medium with *H. pylori*. Following fixation, cells were immediately visualized by confocal microscopy. The number of GFP-LC3B puncta in each cell was counted. HP, *H. pylori*; MOI, multiplicities of infection; CFU, colony forming units

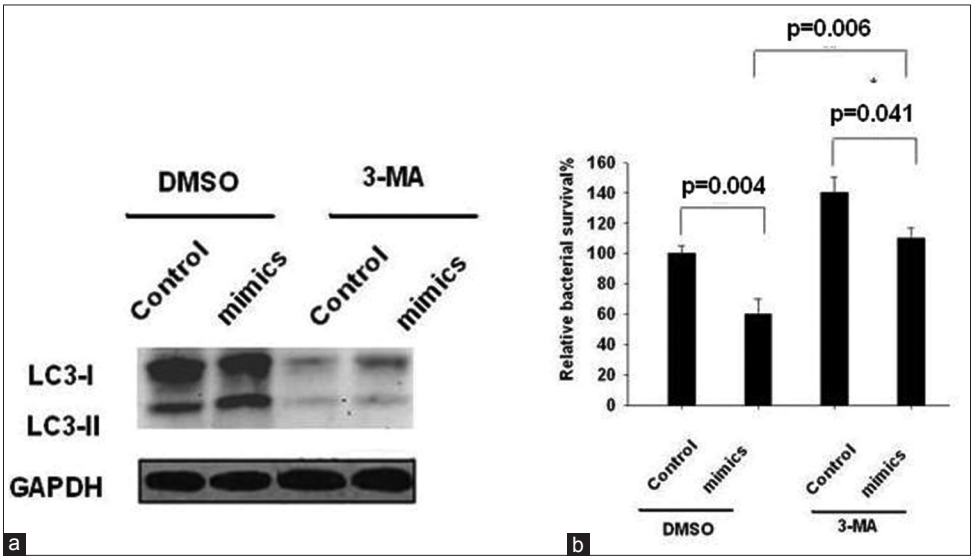

Figure 3: miR-155 decreases the survival of intracellular *Helicobacter pylori* by induction of autophagy. Cells were pretreated with 3-MA, and then transfected with negative control (NC) and miR-155 mimics for 24 h. (a) The ratio of LC3B-II/GAPDH were determined by Western blotting (b) intracellular *H. pylori* viability was determined by CFU counting. MOI, multiplicities of infection; CFU, colony forming units

The results showed that miR-155 mimics obviously decreased the expression of Rheb and RPS6KB2, whereas other target genes did not show significant changes [Figure 4a and b]. We overexpressed Rheb/RPS6KB2 by transient transfecting plasmids pcDNA3.1-Rheb/pcDNA3.1-RPS6KB2 for 24 h following by *H. pylori* infection. As shown in Figure 4c, LC3B-II/GAPDH ratio was sharply reduced in the Rheb and RPS6KB2 overexpressed group, which indicated that Rheb and RPS6KB2 weakened the autophagy development. Cotransfection with microRNA-155 mimics could block this autophagy reduction in the Rheb overexpressed group, but not in the RPS6KB2 group. This suggested that microRNA-155 up-regulated autophagy by suppressing Rheb [Figure 4c and d].

Correlation of miR-155 with clinical value

Then we analyzed the associations between miR-155 and their clinical values in human gastric mucosal tissues. Of the 86 *H. pylori*–positive, chronic gastritis patients, 77 (89.5%) *H. pylori*–infected patients displayed evidence of inflammatory infiltrates with lymphocytes and mononuclear cells, and 19 (22.1%) displayed intestinal metaplasia, whereas the *H. pylori*–negative subjects had mostly normal mucosa. miR-155 was upregulated in *H. pylori*-positive patients [Figure 5a, $P < 0.05$] and was positively correlated with the immunohistochemical grades [Figure 5b]. Furthermore, miR-155 expression was increased in the intestinal metaplasia group [Figure 5c]. We next followed up with eight patients who had showed significant release of *H. pylori* infection before and after the *H. pylori* treatment. Of these patients, six (75%) exhibited significant decreased value of miR-155 [Figure 5c], which indicated that miR-155 could monitor the condition of *H. pylori* infection.

DISCUSSION

H. pylori may promote the development of inflammation of gastric mucosa, which then induces gastric atrophy and intestinal metaplasia, resulting in gastric cancer. This process is a comprehensive action associated with multiple factors, such as inhibition of tumor suppressor genes, overexpression of related genes and a failure to regulate cell proliferation. Studies have revealed that miR-155 participates in various biological pathogenesis of *H. pylori*–related diseases. Xiao *et al*.[7] have demonstrated that miR-155 might be involved in the function as a novel negative regulator to modulate the inflammation response in *H. pylori* infection. Oertli *et al*.[11] have indicated that miR-155 is essential for the T cell-mediated control of *H. pylori* infection. However, whether this microRNA plays a critical role in the regulation of survival of intracellular *H. pylori* remains unknown. Our present results demonstrate that miR-155 decreased the survival of intracellular *H. pylori* at all tested timepoints, which indicated that miR-155 promoted bacterial elimination. As we know, apoptosis is an important biological process for bacterial clearance, but some reports indicate that miR-155 may display a role in the apoptosis inhibition. Saito *et al*.[12] have demonstrated that miR-155 suppress the proapoptotic gene TP53INP1 as their target. Koch's study proposes that the antiapoptotic effects of miR-155 could enhance macrophage resistance to apoptosis induced by DNA damage during *H. pylori* infection.[13] So we hypothesize that there might be a

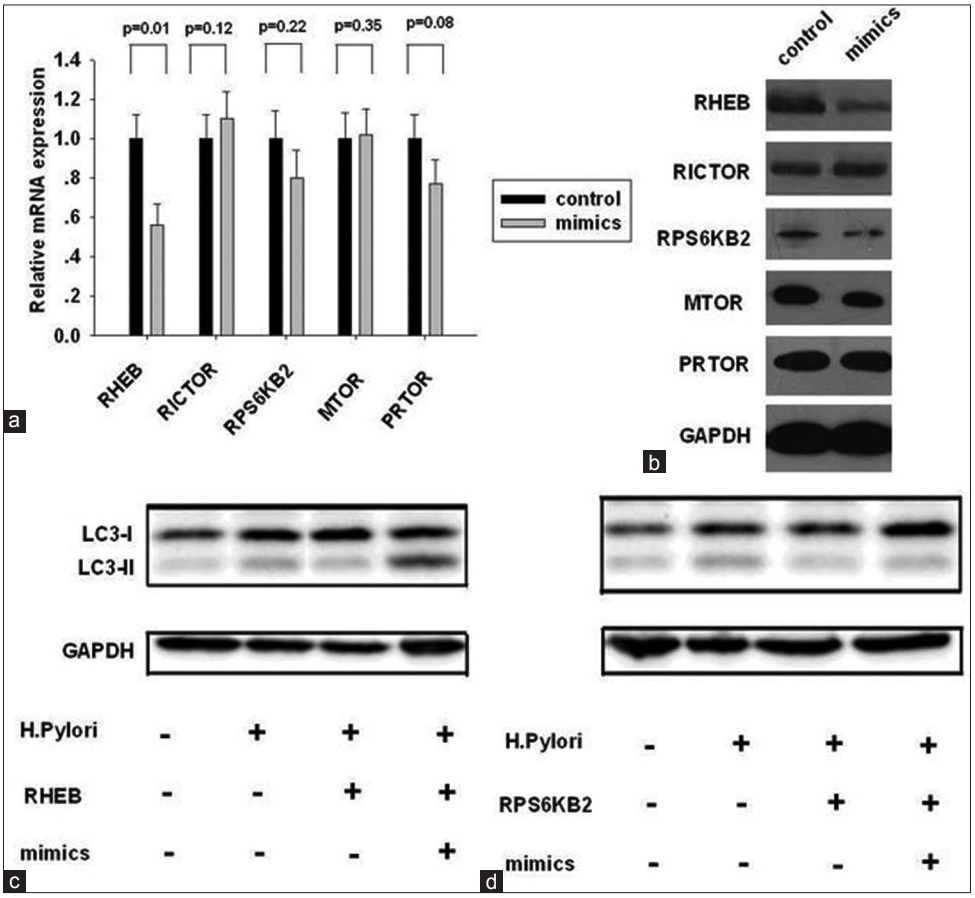

Figure 4: miR-155 induces autophagy by inhibiting Rheb. Cells were transfected with negative control (NC) and miR-155 mimics for 24 h, and (a) mRNA and (b) protein level of Rheb, RPTOR, RICTOR, RPS6KB2, and MTOR were determined. GES-1 cells were transfected with plasmid expressing (c) Rheb or (d) RPS6KB2, together with negative control (NC) or miR-155 mimics, and the ratio of LC3B-II/GAPDH were determined by western blotting

different way for miR-155 to enhance the intracellular *H. pylori* killing.

Autophagy has been demonstrated as a new type of pathogenic bacteria killing mechanism. On testing the autophagy related genes LC3-II, we demonstrated that miR-155 could enhance *H. pylori* induced autophagy. It was shown that blocking autophagy by Rapamycin decreased the intracellular *H. pylori* count, which indicated that miR-155 promoted *H. pylori* clearance through upregulating autophagy. It has become increasingly recognized that altered autophagy is associated with decreasing *H. pylori* survival. Chu *et al*.[14] have reported that the autophagy inducer Rapamycin enhanced the clearance of the *H. pylori*, and *H. pylori* usurp the autophagic vesicles as the site for replication, and the autolysosomes after fusion also degraded the replicating bacteria. Tang *et al*.[6] have demonstrated that *H. pylori* infection increased MIR30B during *in vivo* and *in vitro* infections. Based on the present results, we propose that compromise of autophagy by miR-155 benefits the intracellular *H. pylori* from evading autophagic clearance, which may provide a better understanding of the host anti-*H. pylori* response.

Many genes have been experimentally validated in predicted targets of miR-155, some of which were closely involved in autophagy. Wan *et al*.[10] have reported that miR-155 was an autophagy inducer by targeting multiple players in the MTOR pathway. In this study, we elucidated that miR-155 downregulated the expression of Rheb and RPS6KB2, which were the key proteins in mTOR pathway, playing a key role in promoting autophagy to eliminate intracellular *H. pylori*. Tang *et al*.[15] have identified MyD88 as a novel target of miR-155, and MyD88 was demonstrated targeting Beclin 1 to trigger autophagy.[16] It has suggested that during its co-existence with humans, *H. pylori*, host, and environmental factors consist of a complex network to mediated autophagic processes.

Data of clinical samples showed a significant correlation between miR-155 and immunohistochemical grades in *H. pylori*-positive patients, which confirmed the *in vitro*

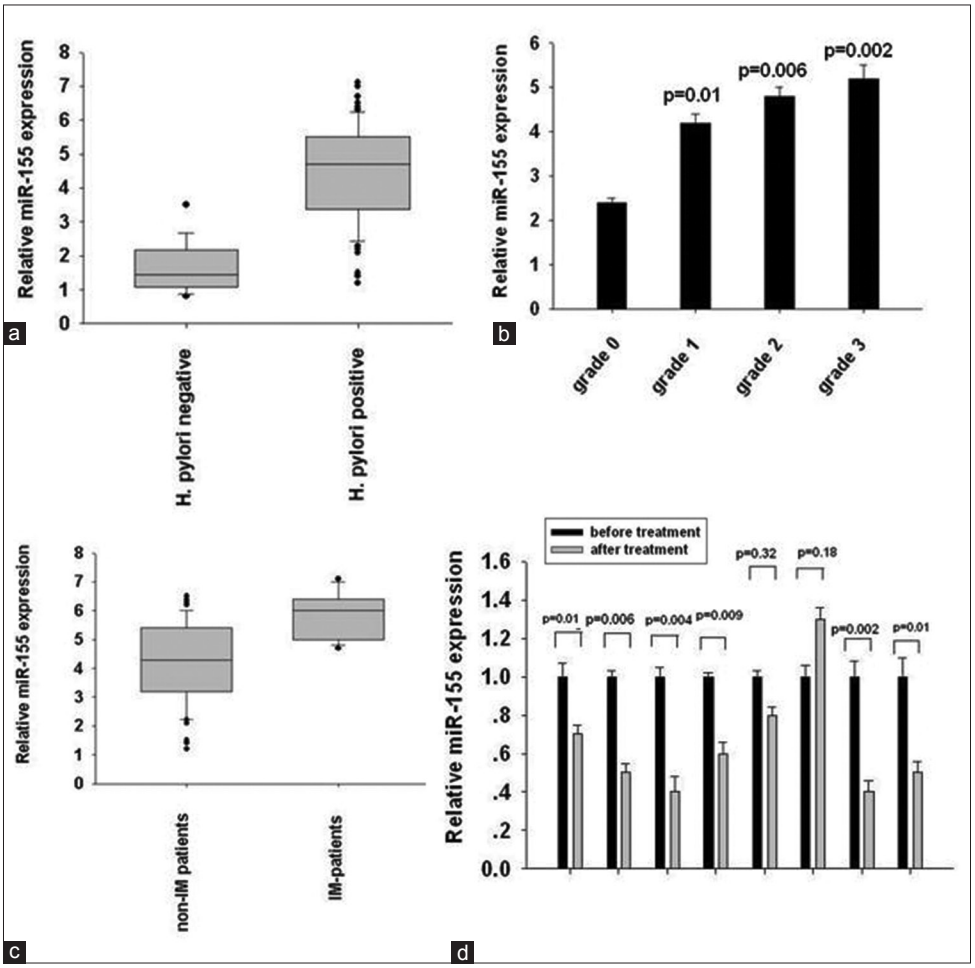

Figure 5: Correlation of miR-155 with clinical value in gastric mucosal tissues. (a) Differential miR-155 expression in gastric mucosal tissues. (b) Correlation of miR-155 and the immunohistochemical grades in gastric mucosal tissues among *Helicobacter pylori* positive patients. (c) Associations between miR-155 and intestinal metaplasia. IM: Intestinal metaplasia. (d) Comparing the differential miR-155 expression in gastric mucosal tissues in 8 patients before and after anti- *H. pylori* treatment

results of our present work. The published data have shown that miR-155 play a potential role as diagnostic and prognostic indicators in various diseases including cancer.[17] When testing the relationship between miR-155 expression and diagnostic index, we found that the miR-155 expression was decreased in the intestinal metaplasia group. Considering that intestinal metaplasia was closely related with gastric cancer, we assumed that miR-155 expression would play a prognostic role in carcinogenesis of *H. pylori*. Our results indicated that miR-155 enhancement might eliminate intracellular *H. pylori* and therefore prevent the gastric cancer occurrence. Molecular mechanism study indicated that this process might through autophagy. In the future, microRNA-based treatments, in combination with traditional chemotherapy, may be a new strategy for the clinical management of *H. pylori*-related gastric cancer.

The main limitation of this work was that the study size is not big enough. Further studies on more patients are ongoing to effect the generalizability of our conclusion. In addition, the relationship between autophagy indicators and clinical index should also be determined.

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