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miR-155 and miR-146b negatively regulates IL6 in *Helicobacter pylori* (cagA+) infected gastroduodenal ulcer

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Abstract. – OBJECTIVE: Helicobacter pylori *(H. pylori)* infection is the main cause of gastroduodenal ulcer. The molecular mechanisms that underlying this progress are still not very clear. MicroRNAs (miRNAs) are small noncoding RNAs that function as negative regulator of numerous target genes at posttranscriptional level. miRNAs plays important roles in the development of many infection related diseases. The roles of miRNAs in the development of *H. pylori*-infected gastroduodenal ulcer haven't been well studied yet.

MATERIALS AND METHODS: The miRNA and mRNA profiles in normal gastroduodenal biopsy, H. pylori-infected gastroduodenal biopsy and H. pylori-infected gastroduodenal ulcer biopsy samples were compared and analyzed to identify potential related miRNAs and their target genes. The differential expression of the identified miRNAs and their target gene were validated in an independent set of *H. pylori* positive gastroduodenal ulcer biopsy samples by immunohistochemistry staining and RT-PCR. Then microRNA mimics were transfected to gastric epithelial cells infected with *H. pylori* 26695 (cagA+). RT-PCR and Western blotting were performed to confirm the target gene of the identified microRNAs.

RESULTS: The integrative analysis and immunohistochemistry staining validation indicated that miR-155 and miR-146b, as well as their predicted target gene IL6, are up-regulated in *H. pylori* positive gastroduodenal ulcer. Further experiments in gastric epithelial cells revealed that *H. pylori* 26695 (cagA+) infection induces IL6 overexpression. But the overexpression of IL6 is weaken due to negative regulation by miR-155 and miR-146b.

CONCLUSIONS: This study indicated that the up-regulation of miR-155 and miR-146b decreases *H. pylori* (cagA+)-introduced IL6 overexpression, which might weaken the cleanup of *H. pylori* (cagA+) and contributes to ulcer.

Key Words:
Gastroduodenal ulcer, miR-155, miR-146b, *H. pylori*, Gene IL6.

Introduction

Helicobacter pylori (H. pylori) is a Gram-negative spiral bacterium that acts as a primary pathogen as its presence in the stomach almost always is associated with a strong mucosal and systemic immune response[1-3]. *H. pylori* infection is one of the most common infectious diseases worldwide, which is estimated to affect 40%-50% population around the world[4]. About 15%-20% of those infected develop severe gastroduodenal diseases, such as peptic ulcer disease (PUD) and gastric cancer (GC)[5-7]. Gastroduodenal ulcer is a distinct breach in the mucosal lining of the stomach (gastric ulcer) or the first part of the small intestine (duodenal ulcer). Almost 100% of patients with gastroduodenal ulcer are infected by *H. pylori*[8], which strongly implies the association between *H. pylori* infection and gastroduodenal ulcer. However, the molecular mechanisms under this association is still not well understood.

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate the expression of target genes via translational repression or mRNA degradation[9]. There are more than one thousand miRNAs in human genome, targeting 30%-60% of all protein-coding genes[10]. They are involved in many important biological processes, including cell growth, differentiation, apoptosis, and organ development[11]. Recent studies have shown relationships between aberrant expression of miRNAs and tumorigenesis[12, 13]. Moreover, there is recent evidence regarding regulatory roles of miRNAs in immune and inflammatory disorders[14, 15]. More and more evidence implies that miRNAs may be involved in the molecular mechanisms that underlies the development of *H. pylori*-infected gastroduodenal ulcer[16].

Thus we compared the miRNA profiles in normal gastroduodenal biopsy, *H. pylori*-infected gastroduodenal biopsy and *H. pylori*-infected gastro-

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duodenal ulcer biopsy samples to investigate the potential miRNAs involving in the development of *H. pylori*-infected gastroduodenal ulcer. Further comparison of mRNA profiles between normal gastroduodenal biopsy and *H. pylori*-infected gastroduodenal biopsy samples were also performed to investigate the potential target genes of miRNAs involving in *H. pylori* infection. The selected candidate miRNAs and mRNAs were validated in an independent set of *H. pylori* positive gastroduodenal ulcer biopsy samples by by immunohistochemistry staining and RT-PCR. Further mechanism investigations were performed in gastric epithelial cells infected with *H. pylori* 26695 (cagA+).

Patients and Methods

Ethics Statement

The study was approved by the Ethical Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. Written informed consents were obtained from all the patients. The entire investigation conforms with the principles outlined in the Declaration of Helsinki.

Patients and Gastroduodenal Samples

Patients undergoing upper gastrointestinal endoscopy in the Second Affiliated Hospital of Xi'an Jiaotong University were included. A total of 18 patients with *H. pylori*-positive (16 cagA+ and 2 cagA-) gastroduodenal ulcer and 9 *H. pylori*-negative control subjects were enrolled. Infection of *H. pylori* was examined by rapid urease test, 13C-urea breath test and histology. Patients were considered to be *H. pylori*-positive when at least two of the tests were positive. Three biopsy samples were obtained from each patient. One sample was used for histologic analysis; one sample for rapid urease test; the final one was frozen into liquid nitrogen immediately for further RNA extraction. Patients with atypical hyperplasia, intestinal metaplasia, atrophic gastritis, systemic infection, liver disease, renal impairment, cancers, use of anti-secretory or non-steroidal anti-inflammatory drugs (NSAIDs), and drugs for *H. pylori* eradication during the preceding month were excluded from the study.

miRNA and mRNA Profile Data Collection

miRNA and mRNA profiles data of normal gastroduodenal biopsy, *H. pylori*-infected gastroduodenal biopsy and *H. pylori*-infected gastroduodenal ulcer biopsy samples are collected from GEO database (www.ncbi.nlm.nih.gov/gds, GSE19769, GSE19769, GSE581). After quality control, 19 normal gastroduodenal biopsy samples, 9 *H. pylori*-infected gastroduodenal biopsy samples (cagA+/-) and 39 *H. pylori*-infected gastroduodenal ulcer biopsy samples (cagA+/-) were used in further miRNA analysis, while 6 normal gastroduodenal biopsy samples and 21 *H. pylori*-infected gastroduodenal biopsy samples were used in mRNA analysis.

Comparison of miRNA and mRNA Profiles

The comparison of miRNA profiles between *H. pylori*-infected gastroduodenal biopsy samples and normal gastroduodenal biopsy samples, as well as the comparison between *H. pylori*-infected gastroduodenal ulcer biopsy samples and *H. pylori*-infected gastroduodenal biopsy samples were performed with Limma package on R platform. The cutline of significant differentially expressed miRNA is Fold Change > 2 and p value < 0.001. The comparison of mRNA profiles between *H. pylori*-infected gastroduodenal biopsy samples and normal gastroduodenal biopsy samples was performed in the same way with the same cutline.

Integrative Network Analysis

The integrative analysis of miRNAs and mRNAs networks was performed with ingenuity pathway analysis (IPA) software, which creates molecular networks of interactions with uploaded genes or miRNA candidates. To build a network and to detect the closest interacting molecules, the IPA utilizes several databases involving direct or indirect molecule relationships. The in-depth analysis of the networks has deciphered the complex interplay of miRNAs and corresponding mRNA targets and suggested their possible roles in the development of *H. pylori*-infected gastroduodenal ulcer.

Cell and H. pylori Culture

The human gastric cancer cell line AGS was obtained from the American Type Culture Collection (Manassas, VA, USA). The AGS cell line was cultured in RPMI-1640 medium and 10% fetal bovine serum at 37°C with presence of 5% CO_2. The wild type H. pylori 26695 (cagA+), which was obtained from American Type Culture Collection, was grown in a micro aerobic humidified atmosphere on agar plate at 37°C with 8% de-fibrinogen goat blood. After 48 hours of culture, *H. pylori* was harvested into the brain heart infusion and then suspended in RPMI 1640 at a concentration of 3×128 colony-forming units (cfu)/ml. *H.*

pylori was then cultured with AGS cells at 100 bacteria/cell. AGS cells were collected at 12 h, 24 h and 48 h after *H. pylori* infection.

Real-time Quantitative PCR

Total RNA including miRNAs and mRNAs were extracted using Trizol reagent (Invitrogen, Grand Islands, NY, USA). RNA concentrations were measured by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). cDNAs were synthesized from enriched total RNA that annealed with random primers and stem-loop reverse transcriptional (RT) primers and then reverse transcribed with MMLV reverse transcriptase (Invitrogen, USA). The synthesized cDNAs were stored at –70°C.

Real-time quantitative PCR was performed using the ABI 7300 Sequence Detection System with primers designed by Primer Express 3.0 and synthesized in Invitrogen (Table I). miRNA levels were normalized by U6 snRNA, while mRNA levels were normalized by GAPDH. All reactions were performed in triplicate.

Immunohistochemistry

Tissue samples for immunohistochemical measurement were fixed in 10% formalin and paraffin-embedded, following routine deparaffinization and rehydration. Incubation with 3% hydrogen peroxide for 10 min was used to inhibit endogenous peroxidase activities. Antigens were retrieved by microwaving in 0.01 mol/L pH 6.0 citrate buffer for 25 minutes, and then cooling to room temperature. After three times of PBS wash, the samples were incubated with anti-human IL6 (AF-206-NA, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Tissue sections were then counterstained with hematoxylin.

Cell Transfection

AGS cells were seeded at 3×105 cells/wells in 6-well plates and incubated overnight. Transfection of the miR-146b miRNA mimic, the miR-55 miRNA mimic, inactive control cel-mir-67 (Thermo Scientific, Dharmacon, Lafayette, CO, USA), or pMIR-Report vectors was performed with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) using 300 nmol of miRNA or 1 μg/ml DNA plasmid, respectively. Total RNAs were extracted at 24 hours after transfection. Total proteins were isolated at 48 hours after transfection.

Table I. Sequence of primers used for real-time PCR.

Name	Primer sequence
IL6	F: 5'-GATGAGTACAAAAGTCCTGATCCA
	R: 5'-CTGCAGCCACTGGTTCTGT
GAPDH	F: 5'-GCCTGGTCACCAGGGCT
	R: 5'-AATTTGCCATGGGTGGAATC
MIR-146B	5'-UGAGAACUGAAUUCCAUAGGCU
MIR-155	5'-ACGCTCAGTTAATGCTAATCGTGATA
U6	5'-TTCCTCCGCAAGGATGACACGC

Western Blot

AGS cells were lysed with RIPA buffer containing a mixture of protease inhibitor cocktail kit for 30 minutes. The lysates were then centrifuged at 4°C and 12,000 rpm for 15 minutes. The protein concentrations were determined by BCA protein assay kit. The proteins were boiled at 95°C for 5 minutes and then stored at –70°C. 50 μg proteins were put to electrophoresis in 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto PVDF membranes. Then PVDF membranes were blocked for one hour in 5% non-fat milk at room temperature. Membranes were probed with antibodies against human IL6 at a dilution of 1:100 at 4°C overnight, following three times wash with TBST. The secondary antibodies were conjugated with horseradish peroxidase and added at a dilution of 1:2000 for one hour. The membranes were washed three times again with TBST and enhanced chemiluminescence was used for detecting antigens on X-film. The β-actin monoclonal antibody (ImmunoCreate, Birmingham, AL, USA) was loaded as the monitor protein a dilution of 1:1000.

Statistical Analysis

The fold changes of gene expression were calculated by the equation $2^{-\Delta\Delta CT}$. Values were expressed as means ± SD. Differences between groups were estimated with *t*-test. All analysis were considered to be significant when p value < 0.05. Statistical analysis was performed using R Statistical Software.

Results

miR-155, miR-146b and IL6 are up-Regulated in Pylori-Infected Gastroduodenal ulcer

The comparison of mRNA and miRNA profiles between *H. pylori*-infected gastroduodenal biopsy samples and normal gastroduodenal

Table II. Functions of mRNAs and miRNAs involved in H. pylori infection.

Name	*p*-value	Molecules
Inflammatory response	1.60E-48 - 8.05E-11	134
Inflammatory disease	7.60E-42 - 8.85E-11	120
Immunological disease	1.03E-41 - 1.08E-10	126
Connective tissue disorders	4.06E-41 - 1.35E-17	89
Skeletal and muscular disorders	4.06E-41 - 1.35E-17	89

biopsy samples identified 230 genes and 35 miRNAs involving in *H. pylori* infection. Among them, 52 genes and 5 miRNAs were up-regulated, while 178 genes and 30 miRNAs were down-regulated during H. pylori infection. Most of these genes and miRNAs are of related functions of immune response (Table II). Down-regulation of these molecules implies suppression of immune response maybe a possible mechanism that underlies chronic *H. pylori* infection.

The comparison of miRNA profiles between *H. pylori*-infected gastroduodenal ulcer biopsy samples and *H. pylori*-infected gastroduodenal biopsy samples found 17 miRNAs were deregulated in *H. pylori*-infected gastroduodenal ulcer. Among them, 13 miRNAs were up-regulated and 4 miRNAs were down-regulated in *H. pylori*-infected gastroduodenal ulcer.

The integrative analysis of miRNAs and mRNAs involving in *H. pylori* infection and *H. pylori*-infected gastroduodenal ulcer indicated that

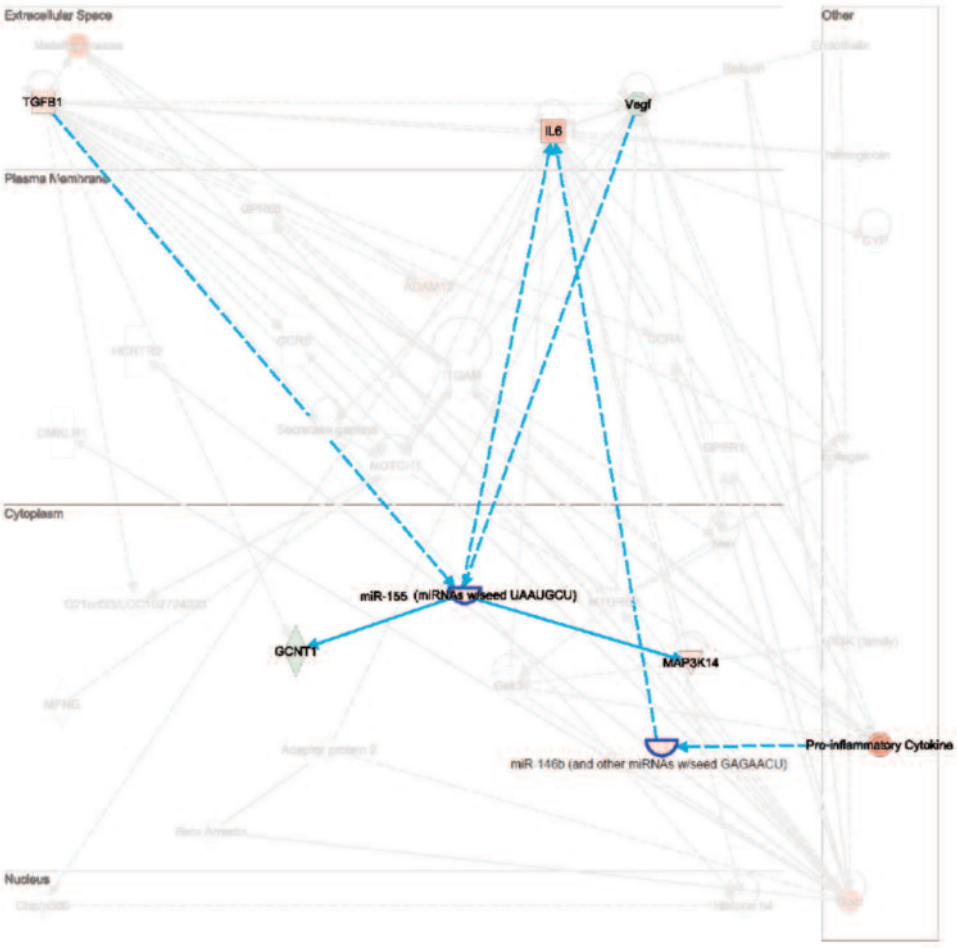

Figure 1. Regulatory networks of mRNAs and miRNAs involved in H. pylori infection. Red means this molecule was up-regulated in H. pylori infection, while green means down-regulated. Blue line indicates the regulatory relationships of miR-155 and miR-146b.

two miRNAs, miR-155 and miR-146b, were up-regulated in the development of *H. pylori*-infected gastroduodenal ulcer (Figure 1). They share a predicted target gene, IL6, an inflammation-related cytokine, which was also up-regulated in *H. pylori* positive samples. This result confused us. However, further investigation in an independent group of 18 *H. pylori* positive (16 cagA+ and 2 cagA-) and 9 *H. pylori* negative gastroduodenal mucosa samples confirmed the up-regulation of miR-155, miR-146b and IL6 in *H. pylori*-infected gastroduodenal ulcer by RT-PCR and immunohistochemistry staining (Figure 2).

Overexpression of IL6 is induced by H. pylori (cagA+) Infection

In order to investigate the potential mechanisms underlying the observed results, we measured the expression of IL6 in a human gastric adenocarcinoma cell line (AGS) after exposure to wild type *H. pylori* 26695 (cagA+) for 12 hours, 24 hours and 48 hours *in vitro*. We found that AGS cells constitutively expressed IL6. *H. pylori* infection induced the increased expression of IL6 mRNA and protein at different time points (Figure 3 A, 3B).

miR-155 and miR-146b Decrease Overexpression of IL6 Induced by H. Pylori (cagA+) Infection

MiRNAs decrease the expression of their target genes through post-transcriptional suppression or mRNA degradation. In order to investigate the mechanisms underlying the observed up-regulation of miR-155, miR-146b and their target gene IL6, we transfected AGS cells with

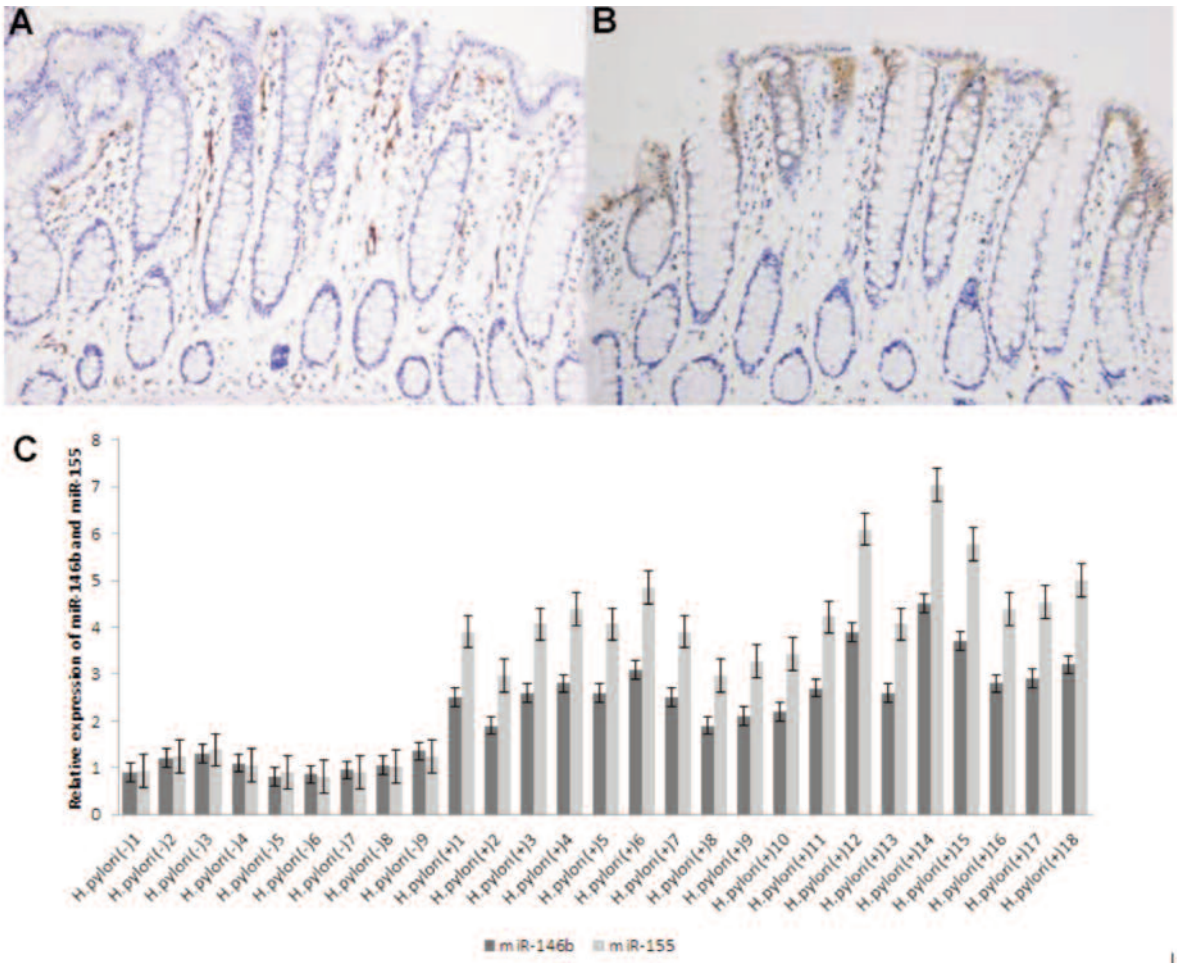

Figure 2. Up-regulation of IL6, miR-155 and miR-146b is confirmed in *H. pylori* positive samples. ***(A)*** immunohistochemistry staining of IL 6 in *H. pylori* positive samples; ***(B)*** immunohistochemistry staining of IL 6 in *H. pylori* negative samples; ***(C)*** Relative expression of miR-155 and miR-146b in gastroduodenal mucosal tissues from H. pylori-positive patients (n = 18) and *H. pylori*-negative normal individuals (n = 9).

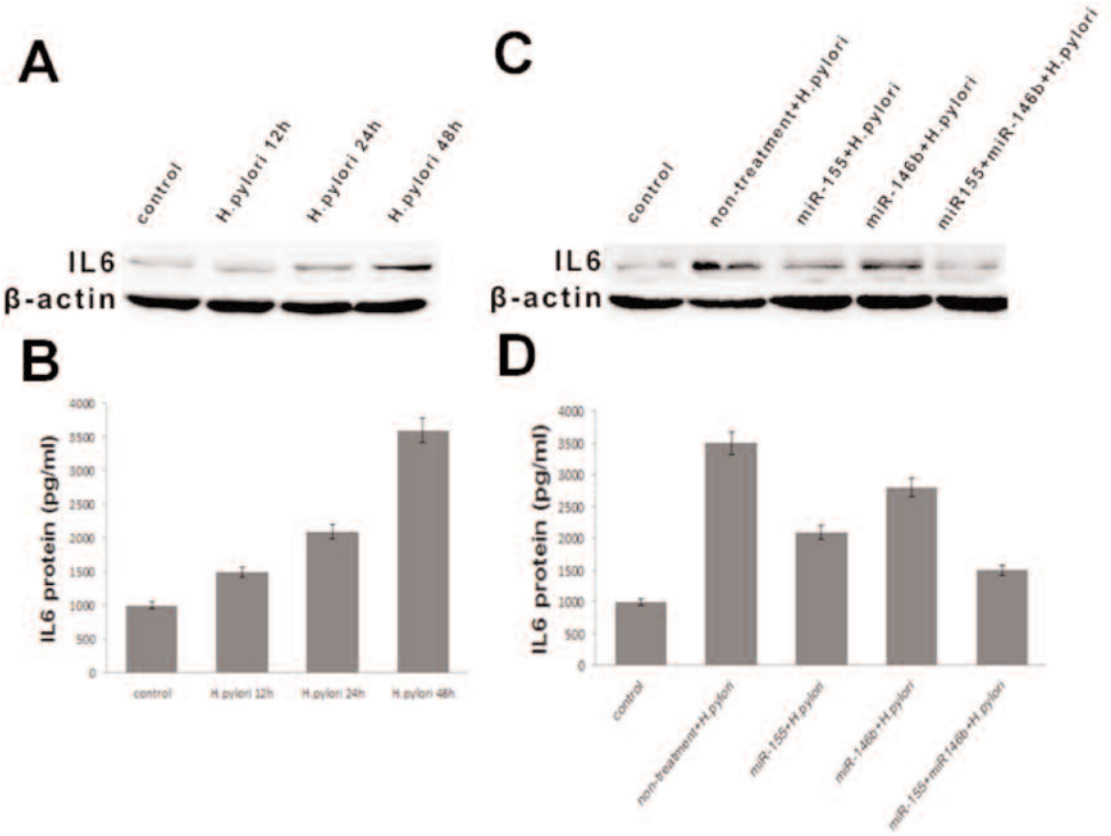

Figure 3. mRNA and protein levels of IL6 in AGS cells after *H. pylori*. ***(A)*** mRNA of IL6 increased with time after *H. pylori* infection; ***(B)*** IL6 protein increased with time after H. pylori infection; ***(C)*** mRNA level of IL6 is negatively regulated by miR-155 and miR-146b; ***(D)*** protein level of IL6 is negatively regulated by miR-155 and miR-146b;

miR-155 and miR-146b, and measured the mRNA and protein levels of IL6. As shown in figure 2, we found a significant increase of IL6 protein in AGS cells with *H. pylori* infection comparing to non-infection. Cells transfected with miR-146b showed a decrease of IL6 mRNA and protein levels compare with non-treatment after *H. pylori* infection. Cells transfected with miR-155 showed a further decrease of IL6 mRNA and protein levels. Moreover, cell transfected with both miR-155 and miR-146b showed the biggest decrease of IL6 mRNA and protein levels, which are only a little higher than *H. pylori* negative controls. It revealed that overexpression of IL6 induced by *H. pylori* Infection is negatively regulated by miR-155 and miR-146b simultaneously.

Discussion

H. pylori plays an essential role in the development of gastroduodenal ulcer[17]. However, the relationships between miRNAs and the immune and inflammatory responses in *H. pylori* infection is not well known. Our study indicated that up-regulated miR-155 and miR-146b decreases the overexpression of IL6 in cagA positive *H. pylori* infected human gastric adenocarcinoma cell line.

The increased expression of miR-155 in *H. pylori* infection has been observed before, and indicated that miR-155 functions as novel negative regulator of the inflammation response to *H. pylori* infection[18]. However, the molecular mechanism of miR-155 in *H. pylori* (cagA+) infection isn't very clear. It's known that miR-146b is an inflammation regulator, which plays an important role on IL6 induced obesity-related inflammatory response[19]. But the role of miR-146b in *H. pylori* (cagA+) infection hasn't be well studied. IL6 is a cytokine that functions in inflammation and the maturation of B cells, which plays important roles in *H. pylori* cleanup. The genetic polymorphisms of IL6 are associated with the development of *H. pylori*-associated gastroduodenal disease[20]. Our study revealed that the overexpres-

sion of IL6 is induced by *H. pylori* (cagA+) infection. However, the up-regulated miR-155 and miR-146b decreases the overexpression of IL6. This impairs IL6 mediated immune response to *H. pylori* infection and results in chronic gastroduodenal inflammation, which might finally leads to ulcer[21].

Conclusions

The up-regulated miR-155 and miR-146b decreases the overexpression of IL6, which impair the immune response to *H. pylori* (cagA+) and contributes to the *H. pylori* (cagA+)-infected gastroduodenal ulcer.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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