Induction of microRNA-155 during *Helicobacter pylori* Infection and Its Negative Regulatory Role in the Inflammatory Response

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**Background.** MicroRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression at posttranscriptional level. *H. pylori* is a major human pathogenic bacterium in gastric mucosa. To date, the role of miRNAs in response to *H. pylori* infection has not been explored.

**Methods.** The expression profile of cellular miRNAs during *H. pylori* infection was analyzed by using microarray and quantitative reverse-transcriptase polymerase chain reaction. The potential target of miR-155 was identified by luciferase assay and Western blot. Promoter analysis and inhibitor experiment were used to investigate the pathway involved in the induction of miR-155. Examination of miR-155 function was performed by overexpression and inhibition of miR-155.

**Results.** *H. pylori* was able to increase the miR-155 expression in gastric epithelial cell lines and gastric mucosal tissues, and nuclear factor–κB (NF-κB) and activator protein–1 (AP-1) pathway were required for the induction of miR-155. miR-155 may down-regulate IkB kinase, Smad-related protein 2, and Fas-associated death domain protein. Furthermore, the overexpression of miR-155 negatively regulated the release of interleukin-8 and growth-related oncogene–α.

**Conclusions.** This study provides the first description of increased expression of miR-155 in *H. pylori* infection, and miR-155 may function as novel negative regulator that help to fine-tune the inflammation response of *H. pylori* infection.

*Helicobacter pylori* is a major human pathogen that is associated with gastric diseases like chronic active gastritis, peptic ulcer, and gastric carcinoma. Approximately 50% of the world’s population is infected with *H. pylori*. A remarkable feature of *H. pylori* infection is its complex and fascinating immune response. Though gastric inflammation is the main mediator of pathology, the immune and inflammatory response is unable to clear the bacterium, resulting in lifelong bacterial persistence. During its long co-existence with humans, *H. pylori*, host and environmental factors consist of a complex network to precisely regulate the immune response. To date, the regulatory mechanism of this complex system is not clear.

MicroRNAs (miRNAs) are small, noncoding RNAs that posttranscriptionally regulate gene expression. Mature miRNAs can specifically bind to 3′ untranslated regions (UTRs) of target cellular mRNA, in turn triggering mRNA degradation or inhibition of translation [1]. To date, hundreds of miRNAs have been identified in the human genome, and miRNAs act as key regulators in a wide variety of biological processes, including development, cell differentiation, apoptosis, metabo-
lism, and signal transduction [2]. Consequently, abnormal patterns of miRNAs have been found in various human diseases, most notably cancer [3].

There has been recent evidence regarding novel role of miRNAs in the regulation of immune system, including the development and differentiation of immune cells, antibody production, and innate immunity regulation [4, 5]. miRNAs are also involved in diseases characterized by abnormal immune response, such as inflammatory or autoimmune disorders [6]. Furthermore, miRNAs have been found to shape the host-virus interactions [7]. These studies provide evidence of a nexus among miRNAs and immune response. However, the potential role of miRNAs in the immune response to a live, whole bacteria is just beginning to be explored.

The aims of our study were to determine whether infection of gastric epithelial cells with H. pylori could lead to the alteration in miRNAs expression, define the underlying mechanism leading to the miRNA up-regulation, and investigate the relationship between miRNAs and H. pylori-induced inflammation.

Materials and Methods

Cell and bacterial culture. GES-1, AGS, MKN45, and human embryonic kidney (HEK) 293 cells were used in our study and were routinely cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum and penicillin (100 U/mL) in a humidified incubator containing 5% CO2 at 37°C. The wild-type H. pylori strains 26695 and 11637 were obtained from ATCC. The wild-type H. pylori clinical isolate strain XN-4 was maintained in our laboratory. All H. pylori strains were grown as described elsewhere [8].

Patients and gastric mucosal specimens. In total, 30 patients undergoing gastroscopy examination at Xinqiao Hospital (Chongqing, China) were included in the study. The patients included 20 patients with H. pylori–induced chronic gastritis (median age, 40 years [range, 25–60 years]; 12 were women, and 8 were men) and 10 H. pylori–negative, healthy control subjects (median age, 35 years [range, 26–55 years]; 6 were women, and 4 were men). The H. pylori infection status was confirmed by bacterial culture, 13C-urea breath test, and his-
H. pylori positive if \( \geq 1 \) of the tests yielded positive results. None of the patients received nonsteroidal anti-inflammatory drugs or had taken antibiotics or proton pump inhibitor drugs in the preceding 4 weeks. The study was approved by the ethics review board at Third Military Medical University, and informed consent was obtained from all patients before participation. Histological assessment was performed according to Sydney classification by 2 pathologists who were blinded to the other experimental results.

**In vitro infection model.** GES-1, AGS, and MKN45 cells were seeded at \( 5 \times 10^6 \) cells/flask 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 morphological changes and the release of interleukin-8 (IL-8) and growth-related oncogene-\( \alpha \) (GRO-\( \alpha \)), as measured by DuoSet ELISA Development System (R&D).

**Microarray analysis of miRNA expression.** Total RNA was isolated using the mirVana RNA Isolation kit (Ambion). RNA samples were sent to CapitalBio (Beijing, China) for miRNA microarray experiments. The miRNA microarray chip contains 924 oligonucleotide probes complementary to mature miRNAs of human, mouse, and rat origin. Raw data were normalized and analyzed with use of GenePix Pro 5.0 software (Axon). Expression data were median-centered by using the global median normalization of the Bioconductor package (http://www.bioconductor.org). Statistical comparisons were done using SAM software, version 2.1 (distributed by Stanford University).

**Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).** qRT-PCR analyses for miRNAs were performed by using TaqMan miRNA assays (Ambion) in a Ro-torgene 6000 Thermocycler (Corbett Life Science). qRT-PCR reactions were performed using the following parameters: 95\(^\circ\)C for 2 min followed by 40 cycles of 95\(^\circ\)C for 15 s and 60\(^\circ\)C for 30 s. U6 small nuclear RNA was used as endogenous control for data normalization. Relative expression was calculated using the comparative threshold cycle method.

qRT-PCR analyses for the mRNA of B cell integration cluster (BIC), IL-8, GRO-\( \alpha \), 1\( \beta \)C kinase \( \varepsilon \) (IKK-\( \varepsilon \)), Smad- and Mad-related protein 2 (SMAD2), and Fas-associated death domain protein (FADD) were performed by using PrimeScript RT-PCR kits (Takara). The mRNA level of \( \beta \)-actin was used as an internal control. The sequences of primers used are shown in Table 1.

**Northern blot.** Northern Blot was performed according the standard procedure, as described elsewhere [8]. The following antisense probes were used: miR-155 (5’-ACCCCTATCAGATAGTGCATTAA-3’) and U6 (5’-ATATCGGACGTTTACAG-TT-3’).

**Plasmid constructs.** The miR-155 promoter report plasmid and mutant plasmids for nuclear factor--\( \kappa \)B (NF--\( \kappa \)B) and activator protein--\( \kappa \) (AP-1) binding sites were kindly provided by Erik Flemington [9]. The construction of luciferase report vectors for miR-155 target, including IKK-\( \varepsilon \), SMAD2, and FADD, was performed according to the instructions and as previously described [10]. Another construct containing mutant seed region was also generated as a control. The sequences of oligonucleotides used are shown in Table 1.

**Luciferase assay.** HEK-293 cells were transfected with 0.8 \( \mu \)g of each firefly luciferase reporter vector, 0.04 \( \mu \)g of Renilla luciferase control vector, pRL-TK (Promega), and 100 nM miR-155 mimics, inhibitors, or scrambled miR-control (Ambion) using Lipofectamine 2000 (Invitrogen). For promoter analysis, GES-1 cells were transfected with report plasmids or miR-155 mimics, followed by \( H. \) *pylori* infection. Luciferase assays were
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Results

Induction of scattering phenotype and proinflammatory cytokine release after H. pylori infection. GES-1 cells were infected with H. pylori 26695 for 24 h. As shown in Figure 1A, morphological changes of cells were observed, and >50% cells exhibited the scattering hummingbird phenotype, which was characterized by spreading and elongation of the cells. The adhesion between H. pylori and GES-1 was observed by scanning electron microscope (Figure 1B). Furthermore, as shown in Figure 1C, H. pylori infection significantly induced the release of proinflammatory cytokines, IL-8, and GRO-α (P < .05). Therefore, our results demonstrated the effectiveness of H. pylori infection of gastric epithelial cells.

miR-155 is up-regulated in response to H. pylori infection. To identify whether miRNAs levels were affected by H. pylori, we measured the miRNAs expression profile by using microarray. This revealed that the expression of miRNAs could be significantly altered during H. pylori infection (Figure 2A). Among the altered miRNAs, PREDICTED_MIR191 was found to be most significant up-regulation (a 16.8-fold change); however, MIR191 was generated from a bioinformatics algorithm and was not validated by experimental methods [11]. Interestingly, some miRNAs involved in the immune response were also increased, including miR-155, miR-16, and miR-146a. To confirm the validity of microarray, a portion of the RNA used for the microarray was subjected to qRT-PCR. Consistent with the microarray findings, the results of qRT-PCR showed that miR-155, miR-16, and miR-146a increased by 3.0-, 2.1-, and 2.5-fold, respectively (Figure 2B). As shown in Figure 2C, the...
Northern blot also identified up-regulation of miR-155 in GES-1 infected with \textit{H. pylori}. The aforementioned findings, together with previous reports of miR-155 on the immune response, prompted us to choose miR-155 for detailed investigation in our study.

**Increased miR-155 level in gastric mucosal tissue specimens from \textit{H. pylori}–positive patients.** To determine whether miR-155 is up-regulated in gastric mucosal tissues after \textit{H. pylori} infection, 20 patients with \textit{H. pylori}–induced chronic gastritis and 10 \textit{H. pylori}–negative healthy control subjects were included in the study. The histological data revealed that most \textit{H. pylori}–infected patients displayed evidence of inflammatory infiltrates with lymphocytes and mononuclear cells, whereas the \textit{H. pylori}–negative subjects had mostly normal mucosa (data not shown). As shown in Figure 2D, miR-155 was highly up-regulated in \textit{H. pylori}–positive patients, with a 3.9-fold increase, compared with the control group (\(P < .05\)). The aforementioned findings documented consistent up-regulation of miR-155 in \textit{H. pylori}–positive gastric mucosal tissues.

**Induction of miR-155 transcription depends on the cell and strain lineage.** It is known that miR-155 is processed from its precursor, BIC [12]. To monitor the kinetics of miR-155 induction, mature miR-155 and BIC mRNA were detected over a 48-h period after \textit{H. pylori} infection. In response to \textit{H. pylori} stimulation, the expression of miR-155 rapidly increased by 2 h, reached its highest levels by 24 h, and slowly decreased by 48 h. BIC mRNA followed a pattern of expression similar to that of miR-155, with the exception of up-regulation occurring at 8-h time points (Figure 3A). To determine whether the same kind of change in miR-155 level could also occur in other gastric epithelial cell lines, we analyzed the miR-155 expression in AGS and MKN45. As shown in Figure 3B, \textit{H. pylori} infection resulted in more notable increase of miR-155 in MKN45 (a 10.3-fold change). This implied that the expression of miR-155 may be tightly regulated and cell-specific. Furthermore, we infected GES-1 cells with 2 additional strains of \textit{H. pylori} (11637 and XN-4). As shown in Figure 3C, compared with 26695 and 11637, the clinical isolate XN-4 up-regulated miR-155 to a lesser extent. XN-4 also induced less IL-8, compared with the other 2 strains (Figure 3D). Taken together, the aforementioned results suggest that the impact of \textit{H. pylori} on the levels of miR-155 appears to depend on the cell and strain lineage, and the induction of miR-155 may be implicated in the pathway of the release of IL-8.

**NF-\(\kappa\)B and AP-1 pathway is required for the induction of miR-155 upon \textit{H. pylori} stimulation.** It has been demonstrated that \textit{H. pylori} stimulation can lead to the activation of NF-\(\kappa\)B and AP-1 in gastric epithelial cells [13]. We transfected

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**Figure 3.** Induction of miR-155 transcription depends on the cell and strain lineage. A, The kinetics of miR-155 and B-cell integration cluster (BIC) induction assayed by quantitative reverse-transcriptase polymerase chain reaction over a 48-h period after \textit{Helicobacter pylori} stimulation of GES-1 cells. B, The miR-155 expression assayed in other gastric epithelial cells, AGS, or MKN-45. C, The miR-155 expression assayed in GES-1 infected with different \textit{H. pylori} strains. D, The release of interleukin-8 (IL-8) protein measured in GES-1 cells infected with different \textit{H. pylori} strains. Data are mean ± standard deviation of triplicate samples and represent 3 separate experiments. *\(P < .05\), compared with noninfected cells.
GES-1 with NF-κB and AP-1 pathway. As shown in Figure 4A, H. pylori was able to significantly activate NF-κB and AP-1 (P < .05). In a previous study, the promoter region of miR-155 was reported to contain putative NF-κB and AP-1 binding sites [14]. To test whether the NF-κB and AP-1 pathway is involved in regulating miR-155 expression, we pretreated GES-1 cells with NF-κB inhibitor (BAY-117082) or JNK inhibitor (SP600125), followed by exposure to H. pylori for 24 h. As shown in Figure 4B, both BAY-117082 and SP600125 blocked miR-155 induction (P < .05), whereas vehicle control had no influence on the miR-155 expression. To further assess the possible contribution of NF-κB and AP-1, the BIC promoter was cloned upstream from a luciferase reporter vector, and the mutant constructs were generated at NF-κB and AP-1 sites. As shown in Figure 4C, mutation of AP-1 site significantly decreased basal promoter activity (P < .05). In contrast, mutation of the NF-κB site had less influence on promoter activity. These findings suggest that both NF-κB and AP-1 pathways are required for the up-regulation of miR-155 in response to H. pylori and that AP-1 plays a central role in the induction of miR-155.

**IKK-ε, SMAD2, and FADD are negatively regulated by miR-155.** To further assess the function of miR-155, it is important to determine which host mRNAs are being regulated by miR-155. Computer analyses using 2 prediction algorithms, TargetScan (http://www.targetscan.org/) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), indicated that transcript coding for IKK-ε, SMAD2, and FADD might be probable targets of miR-155 (Figure 5A). To directly address whether miR-155 binds to the 3′-UTR of target mRNAs, we generated 3 luciferase report vectors that contain the putative miR-155 binding sites within 3′ luciferase report vectors ( ). In contrast, mutation of the NF-κB site had a significant influence on the miR-155 expression. Furthermore, overexpression of miR-155 resulted in the down-regulation of the protein levels of IKK-ε, SMAD2, and FADD, whereas miR-155 inhibitors had no obvious effect on the target proteins (Figure 5E). Taken together, the data above suggest that IKK-ε, SMAD2, and FADD are potential targets of miR-155 and that miR-155 might down-regulate the target protein through different mechanisms.

**Overexpression of miR-155 reduces H. pylori-induced IL-8 and GRO-α.** To provide additional evidence of the role of...
miRNA-155 in negative feedback regulation, we examined the effect of miR-155 on the release of the proinflammatory cytokines IL-8 and GRO-α. As shown in Figure 6A and 6B, miR-155 mimics significantly attenuated the mRNA and protein levels of IL-8 and GRO-α (P < .05). In contrast, miR-155 inhibitors increased *H. pylori*-induced IL-8 and GRO-α to a lesser extent. Next, we tested whether miR-155 attenuated the proinflammatory cytokines through effects on the activity of NF-κB. As shown in Figure 6C, miR-155 mimics significantly diminished NF-κB activity (P < .05), whereas miR-155 inhibitors
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Figure 6. Effect of mimics and inhibitors of miR-155 on Helicobacter pylori–induced interleukin-8 (IL-8) and growth-related oncogene-α (GRO-α) release. A and B, GES-1 cells transfected with miR-155 mimics or inhibitors or scrambled miR-control for 24 h followed by H. pylori infection. The mRNA and protein levels of IL-8 and GRO-α were determined. C, GES-1 cells transfected with NF-κB-Luc or miR-155 mimics or inhibitors for 24 h followed by H. pylori infection. Luciferase activities were normalized to the activity of Renilla luciferase. Results are mean ± standard deviation of triplicate samples and represent 3 separate experiments. *P < 0.05, compared with the control group. D, A proposed model of miR-155-negative feedback regulation of H. pylori–induced inflammation. H. pylori infection induces the up-regulation of miR-155 through NF-κB and AP-1 pathways, which, in turn, can diminish the production of inflammatory cytokines through attenuating NF-κB activity.

had no obvious effect on NF-κB activity. Overall, these results implied that the increase in miR-155 expression during H. pylori infection was involved in the negative feedback regulation of inflammation through attenuating NF-κB activity (Figure 6D).

DISCUSSION

Dysregulation of miR-155 has been associated with several forms of cancer, including lymphoma [15], breast cancer [16], and pancreatic cancer [17]. Interestingly, recent publications have indicated that miR-155 might play a key role in the regulation of normal immunity or inflammation response. Mice deficient in miR-155 have been shown to have defects in the function of T cells, B cells, and dendritic cells [18, 19]. Expression of miR-155 in monocytes or macrophages is also strongly induced by lipopolysaccharide, polyriboinosinic polyribocytidylic acid, CpG, and the cytokine interferon-β [20]. Furthermore, miR-155 is also involved in virus infection and inflammatory disease [21, 22]. The reports above give a strong indication that miR-155 likely plays a central role in the regulation of the response to a broad range of stimuli. In the current study, we report that H. pylori infection can stimulate the expression of miR-155 in gastric epithelial cells and in gastric mucosal tissues. Furthermore, the overexpression of miR-155 can negatively modulate the release of the proinflammatory cytokines IL-8 and GRO-α.

In previous study, potential binding sites for NF-κB and AP-1 have been identified within BIC/miR-155 promoter. Here, we find that the NF-κB and AP-1 pathway is required for the expression of miR-155 induced by H. pylori. However, the pathway involved in miR-155 expression remains controversial. In line with our results, Lam et al [23] suggested that the NF-κB pathway may be implicated in the induction of BIC/miR-155 in lymphoma cells. In addition, Rai et al [24] recently described the overexpression of miR-155 in diffuse large B cell lymphoma, and miR-155–high cell lines showed consistently higher NF-κB activity than did miR-155–low cell lines. In contrast, Van den Berg et al [14] reported that IκB-α-mediated...
blocking of NF-κB did not affect the up-regulation of BIC in Ramos cells. These data add to the complexity of miR-155 regulation.

Recently, it has been reported that TGF-β can induce miR-155 expression through Smad4 Pathway [25]. Moreover, Yin et al [9] reported that c-Ets site had a small but measurable effect in the induction of miR-155. It raises the possibility that there may be differential promoter use in different cell lineages. In addition, Epstein-Barr virus–encoded latent membrane protein 1, which is the major transforming protein of the virus, was able to activate miR-155 transcription through NF-κB pathway [26]. It is well known that H. pylori can use a set of secreted and translocated proteins, including cytotoxin-associated gene A, vacuolating cytotoxin, and outer membrane proteins, to induce downstream signaling pathways, such as AP-1 and NF-κB. Therefore, to look for the specific bacterial elements of H. pylori involved in the induction of miR-155 may be a new challenge.

To date, angiotensin II type I receptor is the first characterized target of miR-155 [27]. Recently, several novel potential target genes have been identified, including activation-induced cytidine deaminase [28]; Ras homolog gene family, member A (Rhoa) [25]; and basic leucine zipper transcription factor 1 [29]. In this report, we identified 3 potential targets of miR-155: IKK-ε, SMAD2, and FADD. As shown in Figure 5E, overexpression of miR-155 resulted in the down-regulation of the target proteins, whereas miR-155 inhibitors had no obvious effect. The reasons may be related to the low level of miR-155 in GES-1 cells. Furthermore, we found that miR-155 may down-regulate the target protein through a different mechanism (either mRNA degradation or inhibition of translation), because miR-155 mimics decreased the mRNA levels of IKK-ε and SMAD2 but not the level of FADD mRNA. It is well known that when miRNA perfectly pairs with the 3’UTR of target mRNA, the target mRNA is usually degraded. On the contrary, when the miRNA shares partial base pairing, it can repress the translation of target mRNA.

Consistent with our results, IKK-ε and FADD have also been found to be potential targets of miR-155 in macrophage [30]. It has been demonstrated that IKK-ε is a novel IκB kinase and that it can regulate NF-κB activation by phosphorylating RelA and c-Rel [31]. Recent studies have indicated that, in addition to its apoptotic function, FADD also plays a role in Toll-like receptor–induced immune responses [32]. According to our report, the capability of miR-155 to control IKK-ε and FADD could possibly lead to limitation of the activation of NF-κB pathway, thus limiting or modulating the inflammation associated with H. pylori infection.

SMAD2 is the primary signaling pathway downstream of transforming growth factor-β (TGF-β). Although the exact mechanism of miR-155 that regulates inflammation by affecting SMAD2 is not clear, considering the induction of TGF-β in H. pylori infection [33], we presume that miR-155 is the negative feedback of the high expression of TGF-β to reduce the pathogenesis of H. pylori infection. Consistent with our speculation, Kong et al [25] have shown that TGF-β can induce miR-155 expression. However, the involvement of miR-155 in the TGF-β/SMAD signaling pathway needs to be further investigated.

Examination of miR-155 function revealed that ectopic overexpression of miR-155 in GES-1 cells attenuated the release of H. pylori–induced IL-8 and GRO-α, whereas miR-155 inhibitors had no obvious effect on IL-8 and GRO-α. The reason for these differences was probably related to the smaller increase of miR-155 in H. pylori infection, compared with the super-maximal concentration that occurs during miR-155 overexpression. Accumulating evidence suggests that H. pylori can lead to the production of proinflammatory cytokines through activation of NF-κB, and potential targets of miR-155—especially IKK-ε and FADD—have been associated with the NF-κB pathway. Therefore, the effect of miR-155 in decreasing the production of IL-8 and GRO-α may be involved in diminishing NF-κB activity (Figure 6C). Consistent with our presumption, Ceppi et al [34] recently showed that miR-155 modulated the TLR/IL-1 signaling pathway by targeting TAB2—an important signal molecule that facilitates activation of TNF receptor–associated factor 6 (TRAF6) and NF-κB. In addition, miR-146 has also been demonstrated to be a negative regulator of NF-κB pathway through the targeting of interleukin-1 receptor–associated kinase 1 (IRAK1) and TRAF6 [35]. Therefore, the effect of miR-155 in modulating inflammation may be a secondary effect that occurs through diminishing NF-κB activity, and the function of miR-155 during H. pylori infection may be more complex than we can imagine. With the increase in our understanding of the role of miR-155, we can expand the information on miR-155 expression in other H. pylori–related diseases, such as peptic ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma.

In conclusion, this is, to our knowledge, the first report to explore the cellular miRNAs expression profile in H. pylori infection. H. pylori can stimulate the expression of miR-155 in gastric epithelial cells and in gastric mucosal tissues, miR-155 may function as a novel negative regulator to modulate the inflammation response in H. pylori infection. Furthermore, altered miR-155 expression may identify a potential link between miRNAs and pathogenesis of H. pylori–related diseases.

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References