

## *H. pylori* related proinflammatory cytokines contribute to the induction of miR-146a in human gastric epithelial cells

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**Abstract** MicroRNAs have been implicated as a central regulator of the immune system. We have previously reported that *Helicobacter pylori* (*H. pylori*) was able to increase the expression of miR-146a, and miR-146a may negatively regulate *H. pylori*-induced inflammation, but the exact mechanism of how *H. pylori* contribute the induction of miR-146a is not clear. Here, we attempted to assess the role of *H. pylori* related proinflammatory cytokines including interleukin (IL)-8, tumor necrosis factor (TNF)- $\alpha$ , and interleukin (IL)-1 $\beta$ , and cytotoxin-associated gene A (CagA) virulence factor on the induction of miR-146a. We found that IL-8, TNF- $\alpha$ , and IL-1 $\beta$  could contribute to the induction of miR-146a in gastric epithelial cell HGC-27 in NF- $\kappa$ B-dependent manner, while the induction of miR-146a upon *H. pylori* stimulation was independent of above proinflammatory cytokines. Furthermore, overexpression of miR-146a reduced *H. pylori*-induced IL-8, TNF- $\alpha$ , and IL-1 $\beta$ . However, CagA had no effect on the miR-146a induction. Taken together, our study suggest that

proinflammatory cytokines IL-8, TNF- $\alpha$ , and IL-1 $\beta$  could contribute to the induction of miR-146a during *H. pylori* infection, while CagA is not necessarily required for miR-146a induction. miR-146a may function as novel negative regulators to modulate the inflammation.

**Keywords** miR-146a · *H. pylori* · Proinflammatory cytokines · CagA

### Introduction

*Helicobacter pylori* (*H. pylori*) is a gram-negative, micro-aerophilic bacterium that can inhabit in the stomach and cause gastritis, peptic ulcer, and gastric cancer. During its long co-existence with humans, host and environmental factors consist of a complex network to regulate the host response. To date, however, the complex regulatory network is not fully understood.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression at the posttranscriptional level. Mature miRNAs can specifically bind to 3'UTRs of target cellular mRNA in turn triggering mRNA degradation or inhibition of translation [1]. miRNAs may act as key regulators in a wide variety of biological processes, including development, cell differentiation, apoptosis, metabolism, and signal transduction [2]. Accumulating evidences have found the regulation role of miRNAs in the innate and adaptive immune response [3–7]. Recent reports of our research and other studies have highlighted the regulatory role of miRNAs in *H. pylori* infection and associated diseases [8]. In our previous study, we first found that *H. pylori* was able to increase the expression of miR-146a, and miR-146a may function as novel negative regulators to fine-tune *H. pylori*-induced inflammation [9]. However, the

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exact mechanism of how *H. pylori* contribute the induction of miR-146a is not clear.

Gastric infection with *H. pylori* is frequently characterized by the predominant infiltration of mononuclear and polymorphonuclear cells, and the accumulation of proinflammatory cytokines, including interleukin (IL)-8, tumor necrosis factor (TNF)- $\alpha$ , and IL-1 $\beta$  [10, 11]. IL-8, a CXC chemokine, plays a pivotal role in the recruitment and activation of neutrophils in response to *H. pylori* infection [12]. The increased gastric mucosal production of IL-1 $\beta$  and/or TNF- $\alpha$  in response to *H. pylori* infection can result in a potent suppression of gastric acid secretion, as well as an enhanced gastric mucosal inflammation [13]. Therefore, IL-8, TNF- $\alpha$ , and IL-1 $\beta$  are strongly linked to the pathogenesis of *H. pylori*. Accumulating evidences have found that TNF- $\alpha$  and IL-1 $\beta$  stimulation can induce the expression of miR-146a in multiple types of cells including monocytes and fibroblasts [14, 15]. However, whether these cytokines can induce the expression of miR-146a in gastric epithelial cells is not clear.

The cytotoxin-associated gene A (CagA) encoded by a 40 kb cag-pathogenicity island (cag PAI) of *H. pylori* has been indicated to play an important role in the pathogenesis in *H. pylori* infection [16]. CagA is the major stimulus of pro-inflammatory cytokine expression in epithelial cells and has the ability to activate NF- $\kappa$ B and induce IL-8 secretion [17, 18]. Additionally, in our previous study, we found that *H. pylori* was able to induce the expression of miR-146a in NF- $\kappa$ B manner. Hence, in current study, in light of the pivotal role of proinflammatory cytokines and CagA, we attempted to assess whether *H. pylori* related proinflammatory cytokines including IL-8, TNF- $\alpha$ , and IL-1 $\beta$ , and CagA virulence factor could contribute to the induction of miR-146a in gastric cells in response to *H. pylori* infection.

## Materials and methods

### Cells and bacterial culture

Human gastric cancer cell line HGC-27 cells were routinely cultured in DMEM medium (Invitrogen) supplemented with 10% fetal calf serum and penicillin (100 U/ml) in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. The wild-type *H. pylori* strain 26695 and 11637 was obtained from ATCC. The  $\Delta$ cagA NCTC11637 *H. pylori* strain was kindly provided by Chihiro Sasakawa [19]. All *H. pylori* strains were grown as previously described [20].

### Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR analyses for miRNAs were performed by using TaqMan miRNA assays (Ambion) in a Rotorgene 6000

Thermocycler (Corbett Life Science). 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. A 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 analyses for the mRNA of IL-8, TNF- $\alpha$  and IL-1 $\beta$  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 were shown in Table 1.

### Cell transfection

All oligonucleotides were synthesized from GenePharma. Transfections were performed using Lipofectamine 2000 (Invitrogen). HGC-27 cells were respectively transfected with 50 nM miR-146a mimics, inhibitors, and scrambled miRcontrol for 24 h or 100 nM IL-8, TNF- $\alpha$  and/or IL-1 $\beta$  small interfering RNA (siRNA) for 48 h. To monitor the transfection efficiency of siRNA, FAM-labeled negative control siRNA was transfected into cells in parallel in all transfections, and at least 80% transfection efficiency was achieved.

### Luciferase assay

For NF- $\kappa$ B activity analysis, HGC-27 cells were cotransfected with 0.8 mg of the reporter luciferase vector pNF- $\kappa$ B-TALuc (Clontech), 0.04 mg of Renilla luciferase control vector, pRL-TK (Promega) with Lipofectamine 2000 (Invitrogen) for 24 h, followed by stimulation of IL-8, TNF- $\alpha$  or IL-1 $\beta$ . Luciferase assays were performed by using the dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

### ELISA

For the determination of cytokines release, supernatants were removed and IL-8, TNF- $\alpha$  and IL-1 $\beta$  levels were measured by DuoSet ELISA Development System (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results are expressed as the means  $\pm$  SEM from triplicate experiments.

### Plasmid constructs

The CagA expression plasmid, pEGFP-C1-CagA, was kindly provided by Chihiro Sasakawa [19].

**Table 1** Sequences of oligonucleotides used in the paper

Name	Sequences
has-miR-146a mimics	5'-UGAGAACUGAAUCCAUGGGUU-3' 5'-CCCAUGGAAUUCAGUUCUCAUU-3'
has-miR-146a inhibitors	5'-AACCCAUGGAAUUCAGUUCUCA-3'
siRNA: IL-8 sense strand	5'-GCCAAGGAGUGCUAAAGAAtdt-3'
siRNA: IL-8 antisense strand	5'-UUCUUUAGCACUCCUUGGC dtdt-3'
siRNA: TNF- $\alpha$ sense strand	5'-GCGUGGAGCUGAGAGAUAAtt-3'
siRNA: TNF- $\alpha$ antisense strand	5'-UUAUCUCUCAGCUCCACGCtt-3'
siRNA: IL-1 $\beta$ sense strand	5'-GGAGAGUGUAGAUCCTCAAAtt-3'
siRNA: IL-1 $\beta$ antisense strand	5'-UUUGGGAUCUACACUCCAG-3'
IL-8-F	5'-GGCAGCCTTCTGATTTCTG-3'
IL-8-R	5'-GGGGTGGAAAGGTTTGGAGT-3'
TNF- $\alpha$ -F	5'-CCCAGGCAGTCAGATCATCTTC-3'
TNF- $\alpha$ -R	5'-GCTTGAGGGTTTGCTACAACATG-3'
IL-1 $\beta$ -F	5'-CATCAGCACCTCTCAAGCAG-3'
IL-1 $\beta$ -R	5'-ATAGCCGTACTCAAAAACCT-3'
$\beta$ -actin-F	5'-TTCCTTCTGGGCATGGAGTCC-3'
$\beta$ -actin-R	5'-TGCGGTACAGGTCTTTGCGG-3'

### Western blot

HGC-27 cells transfected with CagA expression plasmid were washed with ice-cold PBS and then lysed by protein lysate (Pierce). After centrifugation at  $5,000\times g$  for 15 min at  $4^{\circ}\text{C}$ , the protein concentration was measured by BCA protein assay kit (Pierce). Fifty micrograms of lysates were loaded in SDS polyacrylamide 10% gradient gel, and were transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline for 2 h, and were incubated with primary antibodies for GFP (1:1,000, Beyotime) and  $\beta$ -actin (1:1,000, Santa Cruz) monoclonal antibody according to the manufacturer's instructions. The protein of interest was visualized using ECL Western blotting substrate (Pierce) and Chemidoc XRS Gel Documentation System (BioRad).

### Statistical analysis

The results are expressed as mean  $\pm$  S.D. from at least three separate experiments performed in triplicate. The differences between groups were determined using two-tailed Student's *t*-test using SPSS 11.5 software. Statistical differences were declared significant at  $P < 0.05$  level.

## Results

*H. pylori* related proinflammatory cytokines contribute to the induction of miR-146a

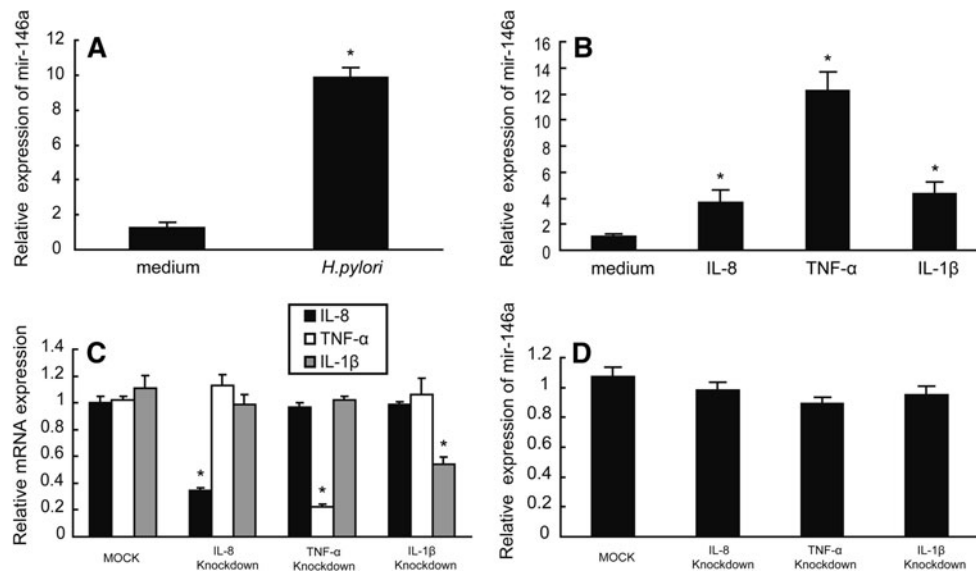
In a previous study, we had shown that miR-146a was significantly up-regulated in many types of gastric epithelial

cells in response to *H. pylori* stimuli. Here we extend this observation to undifferentiated gastric cancer cell line HGC-27. As shown in Fig. 1a, *H. pylori* was able to cause the upregulation of miR-146a in HGC-27 by 9.8-fold ( $P < 0.01$ ). To determine whether *H. pylori* related proinflammatory cytokines could contribute to the induction of miR-146a, we stimulated HGC-27 cells with IL-8, TNF- $\alpha$  and or IL-1 $\beta$  (50 ng/ml) for 8 h and measured the expression of miR-146a by TaqMan qRT-PCR. As shown in Fig. 1b, similar to the stimulatory effects of *H. pylori* infection, IL-8, TNF- $\alpha$  and IL-1 $\beta$  markedly induced miR-146a expression ( $P < 0.01$ ).

To further determine whether IL-8, TNF- $\alpha$  and IL-1 $\beta$  are responsible for miR-146a induction in HGC-27 cells by *H. pylori* infection, we assessed the impact of IL-8, TNF- $\alpha$  and IL-1 $\beta$  silencing by RNA interference. As shown in Fig. 1c, the mRNA levels of IL-8, TNF- $\alpha$  and IL-1 $\beta$  were significantly decreased in HGC-27 cells transfected with siRNAs ( $P < 0.01$ ). Subsequently, HGC-27 cells transfected with siRNAs were infected with *H. pylori* for 24 h, while no significant changes were found on the expression of miR-146a (Fig. 1d). Above results suggest that *H. pylori* related proinflammatory cytokines such as IL-8, TNF- $\alpha$  and IL-1 $\beta$  may induce the expression of miR-146a in gastric epithelial cells, while the induction of miR-146a upon *H. pylori* stimulation is independent of above proinflammatory cytokines.

NF- $\kappa$ B pathway is required for the induction of miR-146a upon stimulation of *H. pylori* related cytokines

Given that NF- $\kappa$ B plays a critical role in the induction of miR-146a, we next investigated whether NF- $\kappa$ B pathway is



**Fig. 1** Up-regulation of miR-146a expression in response to *H. pylori* related cytokines. **a** HGC-27 cells were infected with or without *H. pylori* strain 26695 at MOI 100 for 24 h. The expression of miR-146a were measured by qRT-PCR and normalized to the expression of U6 in each sample. **b** HGC-27 cells was stimulated by *H. pylori* related cytokines such as IL-8, TNF- $\alpha$  and IL-1 $\beta$  (50 ng/ml) for 8 h and the expression of miR-146a were measured by qRT-PCR. **c** HGC-27 cells were transfected with siRNA (100 nM) targeting

IL-8, TNF- $\alpha$  and IL-1 $\beta$  for 48 h. The mRNA levels of IL-8, TNF- $\alpha$  and IL-1 $\beta$  were determined. **d** HGC-27 cells were transfected with siRNA (100 nM) targeting IL-8, TNF- $\alpha$  and/or IL-1 $\beta$  for 48 h followed by *H. pylori* infection. The levels of miR-146a were measured as described in (a). Data are the mean  $\pm$  S.D. (n = 3) of one representative experiment. Similar results were obtained in three independent experiments. \* $P < 0.01$  versus control group

involved in regulating miR-146a expression upon *H. pylori* related cytokines stimulation. As shown in Fig. 2a, IL-8, TNF- $\alpha$  and IL-1 $\beta$  was able to enhance the activity of NF- $\kappa$ B ( $P < 0.01$ ). Furthermore, we pretreated HGC-27 cells with NF- $\kappa$ B inhibitor (BAY-117082), followed by exposure to IL-8, TNF- $\alpha$  and IL-1 $\beta$  for 8 h. As shown in Fig. 2b, BAY-117082 significantly suppressed the miR-146a expression. Above data suggest NF- $\kappa$ B pathway is required for the induction of miR-146a in *H. pylori* related cytokines stimulation.

#### Overexpression of miR-146a reduces *H. pylori*-induced IL-8, TNF- $\alpha$ and IL-1 $\beta$

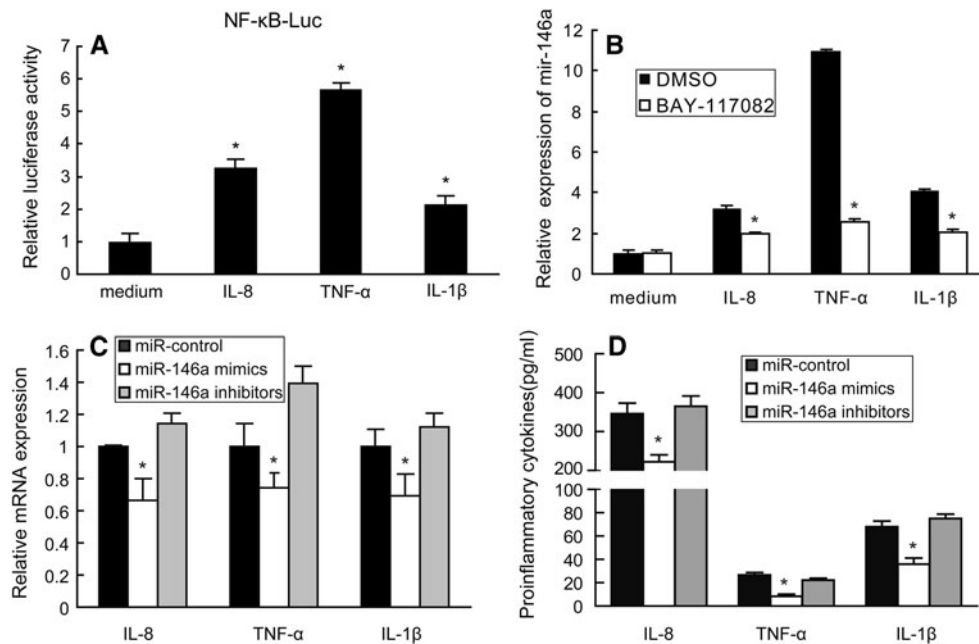
Based on our previous finding that miR-146a was involved in the negative feedback regulation of inflammation during *H. pylori* infection [8, 9], we examined the effect of miR-146a on the release of IL-8, TNF- $\alpha$  and IL-1 $\beta$ . As shown in Fig. 2c, d, when HGC-27 cells were respectively transfected with miR-146a mimics, inhibitors, and miRcontrol for 24 h, followed by *H. pylori* infection, miR-146a mimics significantly attenuated the mRNA and protein levels of IL-8, TNF- $\alpha$  and IL-1 $\beta$  ( $P < 0.01$ ). Overall, above data suggests that the increase of miR-146a may play a potential role in the negative feedback regulation of IL-8, TNF- $\alpha$  and IL-1 $\beta$ .

#### CagA is not necessarily required for miR-146a induction during *H. pylori* infection

CagA, which is one of the key *H. pylori* virulence factors, can be injected into host gastric epithelial cells by type IV secretion system (T4SS) and activate the NF- $\kappa$ B pathway. To determine whether CagA protein is responsible for miR-146a induction during *H. pylori* infection, we compared the miR-146a level in HGC-27 cells infected with wild-type *H. pylori* NCTC11637 and cagA deficient *H. pylori*  $\Delta$ cagA. As shown in Fig. 3a,  $\Delta$ cagA was still able to induce the expression of miR-146a as well as wild-type *H. pylori*. To further test the observation, HGC-27 cells were transfected with CagA expression plasmid, pEGFP-C1-CagA, transfection efficiency was confirmed by Western blot and fluorescence microscopy (Fig. 3b, c). However, expression of exogenous CagA had no effect on the expression of miR-146a. Above data suggests that CagA is not necessarily required for miR-146a induction during *H. pylori* infection.

#### Discussion

Recently, miR-146a has been indicated to play a key role in the regulation of normal immunity or inflammation



**Fig. 2** NF- $\kappa$ B activation is required for the induction of miR-146a upon stimulation of *H. pylori* related cytokines stimulation and overexpression of miR-146a reduces *H. pylori*-induced IL-8, TNF- $\alpha$  and IL-1 $\beta$ . **a** HGC-27 cells were transiently transfected with NF- $\kappa$ B-Luc followed by the stimulation of IL-8, TNF- $\alpha$  and IL-1 $\beta$  (50 ng/ml) for 8 h. Then cells were analyzed by luciferase reporter assay. Luciferase activities were normalized to the activity of Renilla luciferase. **b** HGC-27 cells pretreated with 10  $\mu$ M BAY-117082 prior to the addition of IL-8, TNF- $\alpha$  and IL-1 $\beta$  (50 ng/ml) stimulation. The

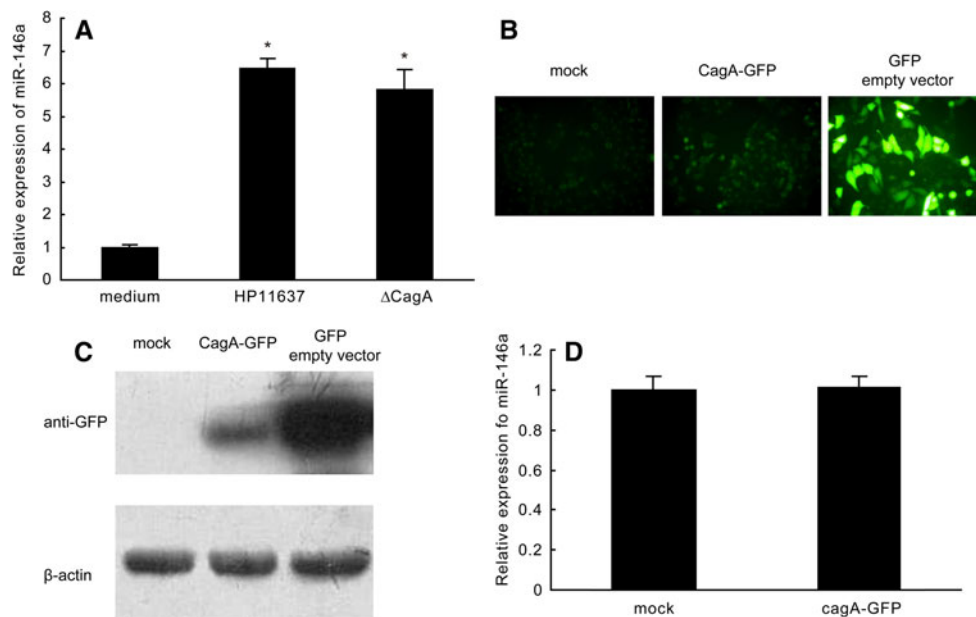
miR-146a expression was assayed by qRT-PCR and normalized to the expression of U6 in each sample. **c** and **d** HGC-27 cells were respectively transfected with scrambled miR-control, miR-146a mimics or inhibitors for 24 h followed by *H. pylori* infection. The mRNA and protein levels of IL-8, TNF- $\alpha$  and IL-1 $\beta$  were determined. Data are the mean  $\pm$  S.D. (n = 3) of one representative experiment. Similar results were obtained in three independent experiments. \* $P$  < 0.01 versus control group

diseases [21, 22]. In our previous research, we reported that *H. pylori* infection caused the elevated expression of miR-146a in gastric epithelial cells. To further investigate the mechanism by which miR-146a were induced by *H. pylori*, we try to explain the roles of proinflammatory cytokines and *H. pylori* virulence factors on the induction of miR-146a. Here, we found that IL-8, TNF- $\alpha$  and IL-1 $\beta$  contributed to the induction of miR-146a in HGC-27 cells in NF- $\kappa$ B dependent manner. Furthermore, overexpression of miR-146a reduced the release of *H. pylori*-induced IL-8, TNF- $\alpha$  and IL-1 $\beta$ . However, CagA was not necessarily required for miR-146a induction during *H. pylori* infection.

A remarkable feature of *H. pylori* infection is increased expression of proinflammatory genes including IL-8, TNF- $\alpha$  and IL-1 $\beta$ . Regarding the possible role of proinflammatory cytokines on the induction of miR-146a, it has been demonstrated that TNF- $\alpha$  and IL-1 $\beta$  stimulation can induce the expression of miR-146a in THP-1 human monocytes and human rheumatoid arthritis synovial fibroblasts [23]. In addition, stimulation with IL-1 $\beta$  resulted in a rapid increase in miRNA-146a in human lung alveolar epithelial cells [14]. In current study, we also observed the induction of miR-146a in gastric epithelial cells treated with IL-8, TNF- $\alpha$  and IL-1 $\beta$ . However IL-8, TNF- $\alpha$  and IL-1 $\beta$  silencing did

not inhibit the induction of miR-146a in HGC-27 cells infected with *H. pylori*. Above results suggest that though *H. pylori* related proinflammatory cytokines may play a role on the expression of miR-146a in gastric epithelial cell, while the induction of miR-146a upon *H. pylori* stimulation is independent of above proinflammatory cytokines.

It is well known that *H. pylori* can use a set of secreted and translocated proteins, including CagA, vacuolating cytotoxin, and outer membrane proteins, to induce downstream signaling pathways [18, 24]. To identify specific *H. pylori* elements responsible for the induction of miR-146a, we first placed our emphasis on an important virulence factor CagA. In our previous study, we have demonstrated that *H. pylori* can stimulate the expression of miR-146a in NF- $\kappa$ B dependent manner. Furthermore, CagA is closely associated with the NF- $\kappa$ B pathway. However, we surprisedly found that CagA deficient *H. pylori* strain also contributed to the induction of miR-146a as well as wild-type *H. pylori* strain, and expression of exogenous CagA could not result in the up-regulation of miR-146a. In line with our results, recent studies have indicated that the mouse-adapted strain Hp76 which does not possess a functional cagPAI also induces miR-155



**Fig. 3** *H. pylori* induces miR-146a expression in CagA independent manner. **a** qRT-PCR analysis of miR-146a expression in HGC-27 cells infected with *H. pylori* strain 11637 (wild type or a cagA-deleted mutant) at MOI 100 for 24 h. The expression of miR-146a was normalized to the expression of U6 in each sample. **b** HGC-27 cells were transfected with 1000 ng of pEGFP-C1-CagA expression plasmids or GFP empty vector for 24 h, fluorescence images of

transfected cells were obtained. **c** GFP protein of transfected cells was detected by western blot to confirm the efficiency of transfection. **d** HGC-27 cells were transfected with pEGFP-C1-CagA expression plasmids or empty vector. The expression of miR-146a was measured by qRT-PCR. Data are the mean  $\pm$  S.D. ( $n = 3$ ) of one representative experiment. Similar results were obtained in three independent experiments. \* $P < 0.01$  versus control group

up-regulation in macrophages [25]. Above data suggests that CagA is not necessarily required for miR-146a induction during *H. pylori* infection.

Examination of miR-146a function in previous study showed that *H. pylori*-induced miR-146a can negatively modulate the inflammation during *H. pylori* infection. Here, overexpression of miR-146a in HGC-27 cells attenuated the release of *H. pylori*-induced IL-8, TNF- $\alpha$  and IL-1 $\beta$ , while miR-146a inhibitors had no obvious effect on these cytokines. The reason for these differences was probably related to the smaller increase of miR-146a in *H. pylori* infection compared to the supermaximal concentrations that occur during miR-146a overexpression. Therefore, miR-146a may play a potential role in the negative feedback regulation of IL-8, TNF- $\alpha$  and IL-1 $\beta$ .

In conclusion, *H. pylori* related cytokines contribute to the induction of miR-146a in gastric epithelial cell. The exact mechanism of how *H. pylori* contribute the induction of miR-146a is complex and needed to be further investigated.

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