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Up-regulation of microRNA-183 reduces FOXO1 expression in gastric cancer patients with *Helicobacter pylori* infection

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Summary. The aim of the study is to detect the expression of FOXO1 mRNA and protein in samples from gastric cancer patients with *Helicobacter pylori* (*H. pylori*) infection, and to investigate the relationship between FOXO1 expression and miR-183 expression. Twenty-six gastric cancer patients with *H. pylori* infection and 26 gastric cancer patients without *H. pylori* infection were included into experimental group and control group, respectively. Tumor tissues and peripheral blood were collected from all subjects. QRT-PCR was used to determine the expression of miRNA and mRNA. Western blotting was carried out to measure protein expression. Dual luciferase reporter assay was used to identify direct interaction between miRNA and 3'-UTR of mRNA. Cell proliferation was examined by CCK-8 assay. FOXO1 mRNA and protein expression was down-regulated in gastric cancer patients, being possibly related to *H. pylori* infection. The expression of miR-183 in tumor tissues and serum from gastric cancer patients with *H. pylori* infection was elevated, and probably regulated the expression of FOXO1 by direct targeting. Stimulation by *H. pylori* up-regulated the expression of miR-183 in gastric cancer AGS cells, and reduced the levels of FOXO1 mRNA and protein. Inhibition of miR-183 elevated the expression of FOXO1 and suppressed the proliferation of AGS cells. The present study demonstrates that the expression of FOXO1 in tumor tissues and blood from gastric cancer patients with *H. pylori* infection is significantly down-regulated, and may be related to the up-regulation of miR-183. *H. pylori* may regulate FOXO1 expression through miR-183 to affect the pathological process of gastric cancer.

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Key words: *Helicobacter pylori*, Gastric cancer, miR-183, FOXO1

Introduction

Gastric cancer is the fifth most prevalent malignant tumor in the world, and it is the third leading cause of cancer-related deaths (Torre et al., 2015). *Helicobacter pylori* (*H. pylori*) infection is considered the first type of carcinogen of gastric cancer by World Health Organization, and high salt and high nitrate diets are also risk factors of the disease (Venerito et al., 2018). Host, environmental factors and bacterial infection ultimately affect the occurence and progression of gastric cancer (Molina-Castro et al., 2017). In developing countries, *H. pylori* infection rate is higher than 90% (Wang, 2014). *H. pylori* infection is an important pathogenic factor of chronic gastritis and gastroduodenal ulcer, being closely related to gastric adenocarcinoma or gastric mucosa-associated lymphoma (Eusebi et al., 2014; Amieva and Peek, 2016). *H. pylori* infection source and transmission route have become important research topics in recent years. In mammals, Akt phosphorylated by PI3K can further phosphorylate FOXO1 and FOXO4 in the FOXO protein family, inhibit their nuclear translocation, and reduce their transcriptional activity in the transcriptional activation of target genes (Kuscu and Celik-Ozenci, 2015). FOXO1 is regarded as an encoded product of tumor-suppressor gene, which may be related to the abnormal mechanism of cell cycle and apoptosis during tumorigenesis. The decrease of FOXO1 activity can shorten the cell cycle, weaken the repair ability after DNA damage, lead to genomic instability, and increase the possibility of tumorigenesis. The lack of FOXO1 protein weakens cell apoptosis and may lead to tumorigenesis. In addition, decreased expression of FOXO protein molecule is also found in tumors with human chromosomal translocation mutations (Song et al., 2019; Ide et al., 2020). In gastric cancer, FOXO1

inhibits the growth of the tumor by inhibiting the self-renewal ability of GC cells through interaction with LGR5 (Choi et al., 2017). FOXO1 is a negative upstream regulator of NF-κB in gastric cancer (Yu et al., 2014). FOXO1 is also an important link between HER2 and MET signaling pathway, as well as a key regulator for acquired Lapatinib resistance of HER2-positive GC cells (Park et al., 2018).

MicroRNAs (miRNAs) are non-encoding RNA with about 22 nucleotides in length, and they play important roles in the pathogenesis of various human diseases by targeting specific mRNA targets (Rupaimoole and Slack, 2017). In cancers, these miRNAs can regulate a large number of protein-coding genes, such as proto-oncogenes and tumor-suppressor genes, to exert their functions in tumors (Saliminejad et al., 2019). Aberrant expression of multiple specific miRNAs has been discovered in gastric cancer (Tian et al., 2014). Several miRNAs have been found to down-regulate FOXO1 in gastric cancer. For example, miRNA (miR)-215 promotes the migration and invasion of gastric cancer cells by targeting FOXO1 (Zang et al., 2017). Up-regulated miR-132 promotes gastric cancer cell growth by inhibiting FoxO1 translation (Li et al., 2016). In addition, miR-96 expression induced by low-dose cisplatin or adriamycin regulates chemosensitivity, cell death and proliferation of gastric cancer SGC7901 cells by targeting FOXO1 (Lang et al., 2018). Our preliminary study predicts that miR-183 is one of the potential upstream regulators of FOXO1. It is reported that abnormal regulation of miR-183 is associated with many types of tumors, including breast cancer and prostate cancer (Lumayag et al., 2013; Leung et al., 2015). The carcinogenesis of miR-183 is regulated by its target genes. For example, Dkk-3 and SMAD4 have been identified as potential target genes of miR-183 (Ueno et al., 2013). In glioblastoma, the up-regulation of miR-183 is significantly correlated with the expression of HIF-1α (Tanaka et al., 2013). However, whether miR-183 can regulate FOXO1 in gastric cancer has not been reported before.

Here, we detect the expression of FOXO1 mRNA and protein in samples from gastric cancer patients with *H. pylori* infection, and try to identify the relationship between FOXO1 expression and miR-183 expression.

Materials and methods

Subjects

For the present study, we chose 26 gastric cancer patients with *H. pylori* infection admitted at our hospital between December 2017 and August 2019 as the experimental group, including 17 males and 9 females (age range, 22-55 years old; median age, 39.8 years old). In the meantime, 26 gastric cancer patients without gastric *H. pylori* infection were included into the control group (19 males and 7 females; age range, 21-57 years old; median age, 39.2 years old). Tumor tissues and blood samples were collected from all subjects. None of the included subjects had intakes of nonsteroidal drugs, proton pump inhibitors, antibiotics, bismuth or alcohol within two weeks before examination.

The subjects underwent ^{13}C urea breath test and gastroscopy, and two pieces of tissues were collected from the antral region of stomachs during gastroscopy. One piece of the tissues was used for rapid urease detection of *H. pylori*, and the other was used for Giemsa staining. Subjects with positive values in at least two of the above three tests were confirmed to have *H. pylori* infection, and those with triple negative values or only one positive value were excluded. All procedures performed in the current study were approved by the Ethics Committee of Jinzhou Medical University. Written informed consent was obtained from all patients or their families.

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from tissues or cells using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), and examined for integrity by gel electrophoresis and purity by ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Scientific, Waltham, MA, USA). Total RNA (1 μg) was used to reverse-transcribe into template cDNA, which was stored at -20°C. The primer sequences for miR-183 were 5'-CGCGGTATGGCACT GGTAGA-3' (upstream) and 5'-AGTGCAGGGTCC GAGGTATTC-3' (downstream). The primer sequences for U6 were 5'-CTCGCTTCGGCAGCACA-3' (upstream) and 5'-AACGCTTCACGAATTTGCGT-3' (downstream). qRT-PCR reaction mixture (20 μl) contained 10 μl qRT-PCR-Mix, 0.5 μl upstream primer, 0.5 μl downstream primer, 2 μl cDNA and 7 μl ddH_2O. The cycling protocol was: initial denaturation at 95°C for 5 min; denaturation at 95°C for 15 sec, annealing at 57°C for 25 sec and elongation at 72°C for 30 sec (46 cycles). The results were analyzed by $2^{-\Delta\Delta Ct}$ method and the ratio of miR-183/U6 was calculated.

The primer sequences for FOXO1 were 5'-ACGAG TGGATGGTCAAGAGC-3' (upstream) and 5'-AATTG AATTCTTCCAGCCCGC-3' (downstream). The primer sequences for GAPDH were 5'-AGGAGCGAGACCCC ACTAACAT-3' (upstream) and 5'- GTGATGGCATGG ACTGTGGT-3' (downstream). qRT-PCR reaction mixture (20 μl) contained 10 μl qRT-PCR-Mix, 0.5 μl upstream primer, 0.5 μl downstream prime, 2 μl cDNA and 7 μl ddH_2O. The cycling protocol was: initial denaturation at 95°C for 5 min; denaturation at 95°C for 25 sec, annealing at 57°C for 20 sec and elongation at 72°C for 30 sec (46 cycles). The results were analyzed by $2^{-\Delta\Delta Ct}$ method and the ratio of FOXO1/GAPDH was calculated.

Cell culture and transfection

293T cells and AGS cells were purchased from the

Type Culture Collection, Chinese Academy of Sciences, Shanghai, China, and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) under 37°C and 5% CO_2. On the day before transfection, cells in logarithmic growth phase (3×10^5) were seeded onto 24-well plates containing antibiotics-free RPMI-1640/10% FBS. When 70% confluence was reached, 0.5 μg plasmids/agomiR (antagomiR-183, 5'-AGUGAAUUCU ACCAGUGCCAUA-3'; antagomiR-NC, 5'-CAGUACU UUUGUGUAGUACAA-3'; agomiR-183, 5'-CCGCAG AGUGUGACUCCUGUUCUGUGUAUGGCACUGGU AGAAUUC-3'; agomiR-NC, 5'-UUUGUACUACACA AAAGUACUGCAGUACUUUUGUGUAGUACAAA-3'; Sangon, Shanghai, China) was mixed with 50 μL Opti Mem medium (Thermo Fisher Scientific, Waltham, MA, USA) in a vial, and 1 μL Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was mixed with 50 μL Opti Mem medium in another vial. Five minutes later, the contents in the two vials were mixed together and kept under room temperature for 20 min before being added into the cells. Six hours later, the medium was changed to fresh RPMI-1640/10% FBS and the cells were cultured for 48h. Then, the cells were collected for further assays.

Bioinformatics

Bioinformatics prediction is a powerful tool for the study of the functions of miRNAs. We used TargetScan 7.2 (http://www.targetscan.org/vert_72) to predict target genes that might regulate FOXO1 gene.

Dual luciferase reporter assay

According to bioinformatics results, wild-type (WT) and mutant seed regions of miR-183 in the 3'-untranslated region (UTR) of FOXO1 gene were chemically synthesized in vitro. Then, their two ends were attached with Spe-1 and HindIII restriction sites, and then cloned into pMIR-REPORT luciferase reporter plasmids. Plasmids (0.8 μg) with WT or mutant 3'-UTR sequences were co-transfected with agomiR-183 (100 nM; Sangon Biotech, Shanghai, China) into 293T cells using Lipofectamine 2000 as described above. For control, 293T cells were transfected with agomiR-negative control (NC). After cultivation for 24h, the cells were lysed using dual luciferase reporter assay kit (E1980; Promega, Fitchburg, WI, USA) according to the manufacturer's manual, and luminescence intensity was measured using GloMax 20/20 luminometer (Promega, Fitchburg, WI, USA). Using renilla luminescence activity as internal reference, the luminescence values of each group of cells were measured.

H. pylori culture and infection

H. pylori 11637 (wild-type *H. pylori*) strain was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in 14 infusion boards containing 14% rabbit blood under microaerobic conditions and 37°C (5% O_2, 10% CO_2 and 85% N_2). Before infection, cells were inoculated in 24-well plates until 80% confluence. Then, the cells were incubated with RPMI-1640 medium containing *H. pylori* [multiplicities of infection (MOI)=100] for 48h before assays.

Western blotting assay

Total proteins were extracted from samples and BCA method (P0011; Beyotime, Shanghai, China) was used to measure protein concentrations. The samples were boiled with SDS-PAGE loading buffer for 5 min. Then, 20 μg protein samples underwent 10% SDS-PAGE, and were transferred onto PVDF membrane on ice at 100V for 2h. After being blocked with 5% non-fat milk at room temperature for 1h, the membranes were incubated with rabbit anti-human FOXO1 polyclonal primary antibody (1:500 dilution; ab39670; Abcam, Cambridge, UK) and rabbit anti-human β-actin polyclonal primary antibody (1:3000 dilution; ab129348; Abcam, Cambridge, UK) at 4°C overnight. Then, the membranes were incubated with goat anti-rabbit secondary antibody (1:5000 dilution; ab6721; Abcam, Cambridge, UK) at room temperature for 1h. The membranes were then soaked in enhanced chemi-luminescence reagent (ab65623; Abcam, Cambridge, UK), and imaged. Image lab software (v3.0; Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative expression of target protein was expressed against β-actin.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged at 3000 rpm for 10 min before separating serum, which was used for ELISA test. Cell culture supernatants were directly used for ELISA test. ELISA was performed following the manufacturer's protocols. Within 15 min after adding stop solution, the absorbance at 450 nm of each well was read.

Cell proliferation assay

Cells were seeded into 96-well plates at a density of 2×10^3 cells per well. Each condition was tested in triplicate wells. At 24, 48 and 72h, 20 μl MTT (5 g/l; JRDC000003, JRDUN Biotechnology, Shanghai, China) was added to each well, followed by incubation for 4h at 37°C. After aspiration of medium, DMSO (150 μl per well) was added to dissolve purple crystals. Then, the absorbance of each well was measured at 490 nm with a microplate reader (Bio-Rad, Hercules, CA, USA) and cell proliferation curves were plotted.

Statistical analysis

The results were analyzed using SPSS 18.0

statistical software (IBM, Armonk, NY, USA). The data were expressed as means ± standard deviations. Data were tested for normality. Multigroup measurement data were analyzed using one-way ANOVA. In case of homogeneity of variance, Least Significant Difference and Student-Newman-Keuls methods were used; in case of heterogeneity of variance, Tamhane's T2 or Dunnett's T3 method was used. Comparison between two groups was carried out using unpaired Student's t-test. $P<0.05$ indicated statistically significant differences.

Results

Down-regulation of FOXO1 mRNA and protein in gastric cancer patients is possibly related to H. pylori infection

FOXO1 is one of the factors that inhibit the growth of gastric cancer, but its relationship with *H. pylori*, the primary carcinogen of gastric cancer, is unknown. Therefore, we first examined the expression of FOXO1 in gastric cancer tissues and blood from gastric cancer patients. Compared with control group, the expression of both mRNA and protein of FOXO1 in tumor tissues and serum from gastric cancer patients with *H. pylori* infection was markedly reduced ($P<0.05$) (Fig. 1). The result indicates that the down-regulation of FOXO1 mRNA and protein in gastric cancer patients is possibly related to *H. pylori* infection.

miR-183 possibly regulates the expression of FOXO1 by direct targeting

Using bioinformatics prediction, we found that miR-183 is a potential regulator of FOXO1 gene (Fig. 2A). Furthermore, qRT-PCR showed that the expression of miR-183 in tumor tissues and serum from gastric cancer patients with *H. pylori* infection was significantly higher than that in gastric cancer patients without *H. pylori* infection (Fig. 2B,C). Dual luciferase reporter assay elucidated that the transfection by agomiR-183 directly reduced the relative fluorescence intensity of the cells transfected by plasmid inserted with FOXO1 sequence ($P<0.01$) (Fig. 2D). The results suggest that miR-183 possibly regulates the expression of FOXO1 by direct targeting.

The up-regulation of miR-183 in AGS cells stimulated by H. pylori significantly reduces the levels of FOXO1 mRNA and protein

To examine the *in vitro* effect of *H. pylori*, we used *H. pylori* (MOI=100) to stimulate AGS cells and measured the expression of miR-183 and FOXO1 in the cells. Our observation showed that *H. pylori* treatment markedly elevated the expression of miR-183 (Fig. 3A), but significantly decreased the levels of FOXO1 mRNA and protein in AGS cells (Fig. 3B,C).

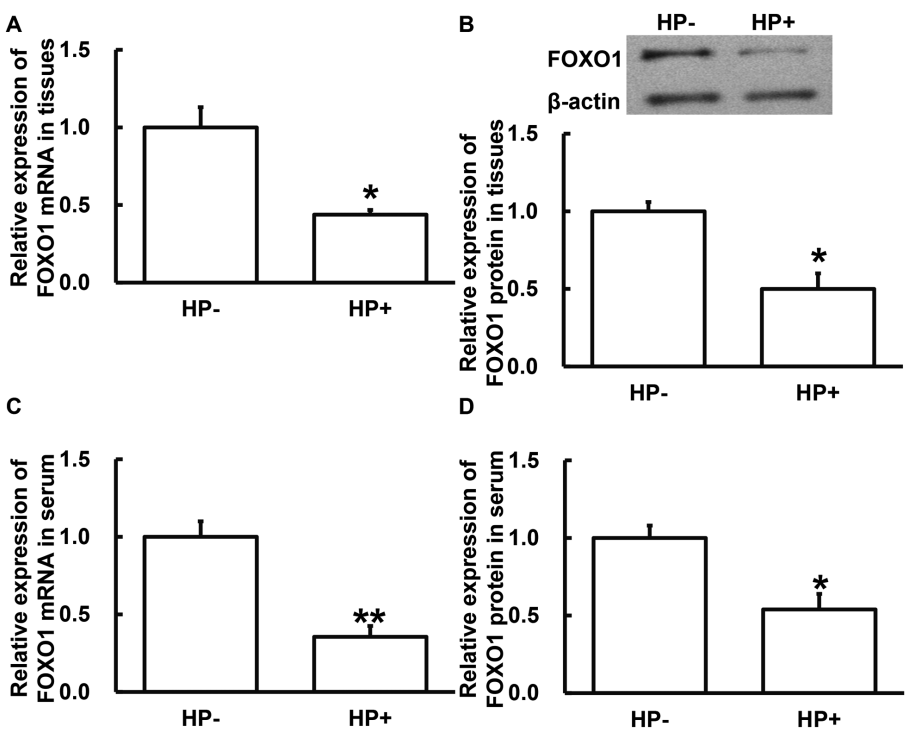

Fig. 1. Expression of FOXO1 in gastric cancer patients with/without *H. pylori* infection. **A, B.** Relative expression of FOXO1 **(A)** mRNA and **(B)** protein in tumor tissues from gastric cancer patients with/without *H. pylori* infection. **C, D.** Relative expression of FOXO1 **(C)** mRNA and **(D)** protein in serum from gastric cancer patients with/without *H. pylori* infection. HP-, gastric cancer patients without *H. pylori* infection; HP+, gastric cancer patients without *H. pylori* infection. *P<0.05 and **P<0.01 compared with HP- group.

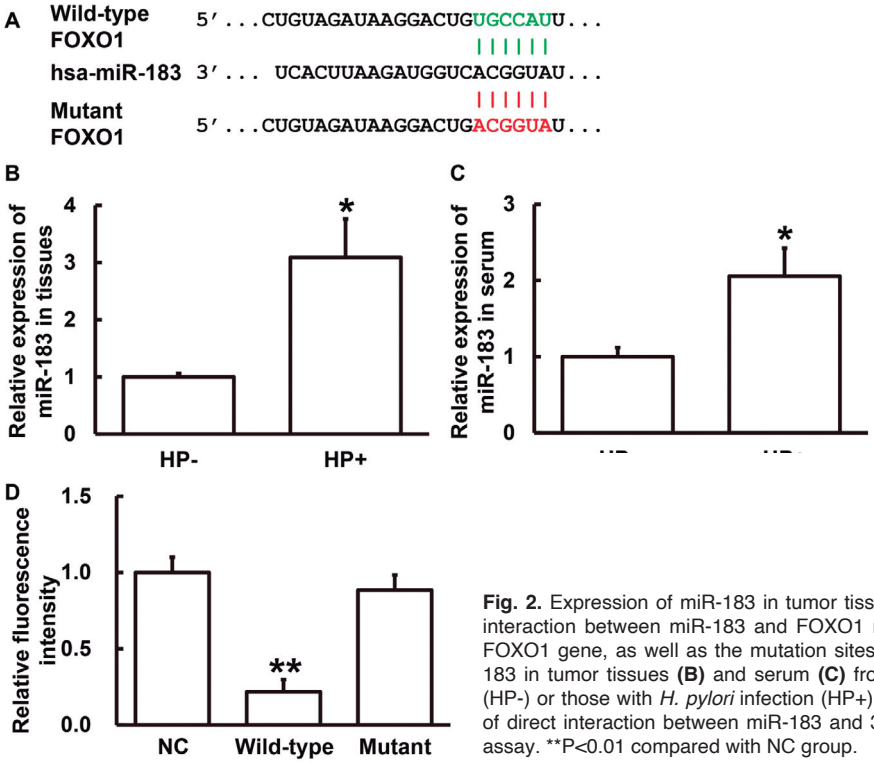

Fig. 2. Expression of miR-183 in tumor tissues and serum from gastric cancer patients and the interaction between miR-183 and FOXO1 mRNA. **A.** The binding sites between miR-183 and FOXO1 gene, as well as the mutation sites in FOXO1 gene. **B, C.** Relative expression of miR-183 in tumor tissues **(B)** and serum **(C)** from gastric cancer patients without *H. pylori* infection (HP-) or those with *H. pylori* infection (HP+). *P<0.05 compared with HP- group. **D.** Identification of direct interaction between miR-183 and 3'-UTR of FOXO1 mRNA by dual luciferase reporter assay. **P<0.01 compared with NC group.

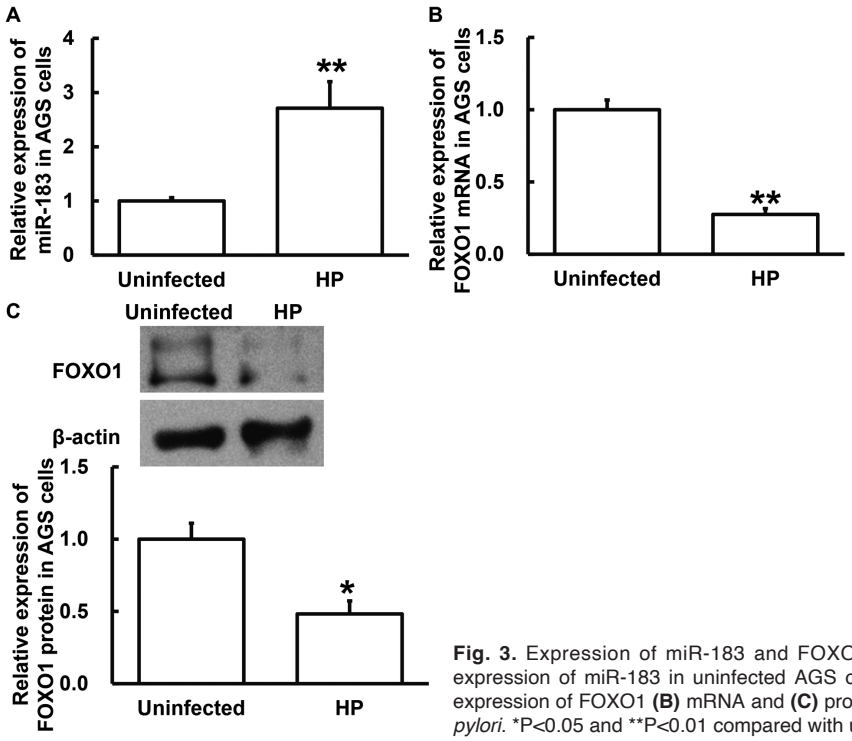

Fig. 3. Expression of miR-183 and FOXO1 in AGS cells stimulated by *H. pylori*. **A.** Relative expression of miR-183 in uninfected AGS cells and those stimulated by *H. pylori*. **B, C.** Relative expression of FOXO1 **(B)** mRNA and **(C)** protein in uninfected AGS cells and those stimulated by *H. pylori*. *P<0.05 and **P<0.01 compared with uninfected group.

Inhibition of miR-183 elevates the expression of FOXO1 and suppresses the proliferation of gastric cancer AGS cells.

Then, we transfected antagomiR-183 into AGS cells and qRT-PCR showed inhibited miR-183 expression in these cells (Fig. 4A). Moreover, the expression of FOXO1 mRNA and protein in AGS cells transfected with antagomiR-183 was significantly higher than that in antagomiR-NC group ($P<0.05$) (Fig. 4B,C). Cell proliferation assay showed that AGS cells transfected with antagomiR-183 had weaker proliferation activity than those in antagomiR-NC group ($P<0.05$ at 72h) (Fig. 4D). The results elucidate that inhibition of miR-183 elevates the expression of FOXO1 and suppresses the proliferation of gastric cancer AGS cells.

Discussion

H. pylori was first isolated from gastric parenchyma of patients with gastritis in 1983 (Ianiro et al., 2015). Barry J. Marshall and J. Robin Warren pointed out for the first time that *H. pylori* may be an important pathogenic factor for diseases such as active chronic gastritis, duodenal disease and gastric ulcer (Pincock, 2005). The gene changes in gastric epithelial cells stimulated by *H. pylori* is one of the important inducements of many gastric diseases. For example, *H. pylori* infection affects the proliferation and apoptosis of gastric epithelial cells and fibroblasts (Gonciarz et al., 2019). Our observations in the present study confirmed that compared with gastric cancer patients without *H. pylori* infection, the expression of FOXO1 mRNA and protein in tumor tissues and serum of gastric cancer patients with *H. pylori* infection was significantly down-regulated.

Previous studies have shown that in breast cancer (Huang and Ling, 2017), pancreatic cancer (Moeinifard et al., 2017) and ovarian cancer (Gao et al., 2012), FOXO1 can interact with downstream proteins Bim and P27 to affect tumor formation (Ju et al., 2014). FOXO family members include FOXO1, FOXO3, FOXO4 and FOXO6 proteins, which play important roles in mammalian cell apoptosis, stress, cell cycle arrest and DNA damage/repair (Katoh and Katoh, 2004). FOXO1 has been considered as a classic tumor-suppressor gene, and the down-regulated expression or nuclear loss of FOXO1 have been found in some tumors (Song et al., 2019; Yan and Huang, 2019). In prostate cancer, FOXO1 has been reported to directly inactivate Runx2 transcriptional activity, thereby inhibiting tumor cell migration and invasion (Zhang et al., 2011). In prostate cancer, up-regulated expression of MAOA activates neuropilin-1 in a dependent manner to induce AKT phosphorylation, decrease the expression of FOXO1 in the nucleus, reduce the binding with TWIST promoter and increase TWIST expression, thus promoting the metastasis of cancer cells (Wu et al., 2014).

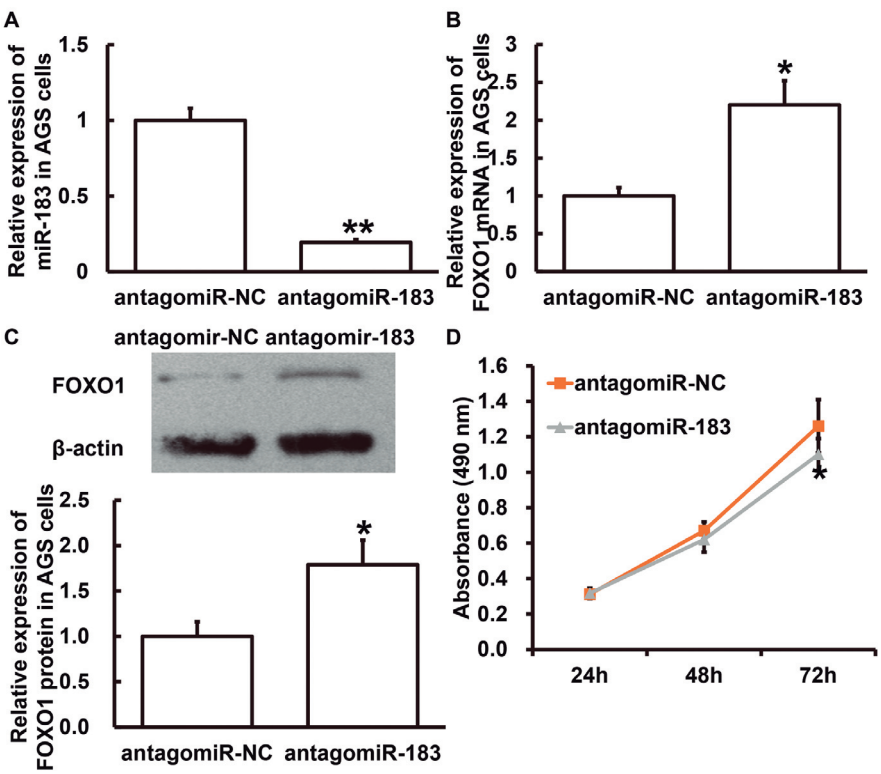

Fig. 4. Effect of miR-183 inhibition on FOXO1 expression and the proliferation of AGS cells. **A.** Relative expression of miR-183 in AGS cells transfected with antagomiR-NC or antagomiR-183. **B, C.** Relative expression of FOXO1 **(B)** mRNA and **(C)** protein in AGS cells transfected with antagomiR-NC or antagomiR-183. **D.** Proliferation of AGS cells transfected with antagomiR-NC or antagomiR-183. *$P < 0.05$ and **$P < 0.01$ compared with antagomiR-NC group.

In order to further study the regulatory mechanism of FOXO1, we used bioinformatics to predict the upstream genes of FOXO1. miR-183 is one of the upstream factors that we predicted to regulate FOXO1. It is reported that miR-183/182/96 family is up-regulated in renal cell carcinoma, colorectal cancer and breast cancer (Zhang et al., 2015; Song et al., 2016; Yuan et al., 2019). Moreover, miR-183 family gene amplification is also found in melanoma cells (Sun et al., 2017). These studies suggest that the miR-183 family can promote the occurrence and development of tumors as an oncogene. Abundant experiments show that the miR-183 family plays the role of proto-oncogenes in different types of tumors. A study shows that miR-183 can reduce the apoptosis of hepatoma cells by inhibiting the expression of PDCD4 at the level of gene translation (Li et al., 2010). It is also shown that EGR1 is regulated by miR-183 in tumors, and inhibition or knockout of miR-183 in tumor cells can significantly increase the expression of EGR1 and PTEN proteins and affect cell migration (Sarver et al., 2010). There are also some studies on the role of miR-183 family as tumor-suppressor genes. For example, Ezrin protein plays important roles in the invasion and metastasis of lung cancer and breast cancer. VIL2, the gene encoding Ezrin protein, is one of the downstream target genes of miR-183. Overexpression of miR-183 reduces the expression of the VIL2 gene, and ultimately inhibits the migration and invasion of lung cancer and breast cancer cells (Wang et al., 2008; Lowery et al., 2010). Pomegranate extract can inhibit the proliferation and promote the apoptosis of MCF-7 breast cancer cells. In this process, the expression of miR-183 is increased significantly, and down-regulates the expression of RAD50 (Shirode et al., 2014). The above studies confirm the abnormal expression of miR-183 family in different tumors, and also explain part of the mechanism of miR-183 family in promoting and inhibiting different tumors. In our experiments, we discovered that contrary to the trend of FOXO1 expression, the expression of miR-183 was significantly up-regulated in gastric cancer patients with *H. pylori* infection, which was consistent with the regulation pattern of microRNA and mRNA to some extent. Furthermore, our dual luciferase reporter assay confirmed that miR-183 was able to directly bind to the 3'-UTR of FOXO1 mRNA. Therefore, we hypothesized that miR-183 might also be related to the stimulation by *H. pylori*. Subsequently, we stimulated AGS cells by *H. pylori* and examined the expression of miR-183 and FOXO1. Our results showed elevated expression of miR-183 and decreased expression of FOXO1, further indicating the regulation of miR-183/FOXO1 by *H. pylori*. The *in vitro* transfection experiments in the end confirmed that the direct inhibition of miR-183 expression in gastric cancer AGS cells led to the up-regulation of FOXO1 expression, and resulted in significantly reduced proliferation activity of the cells. The result demonstrated the important biological role of the miR-183/FOXO1 axis in gastric cancer cells.

In addition to conventional tissues for tumor study, we also selected blood samples. Interestingly, the expression trend of miR-183/FOXO1 axis in the blood was consistent with that in tissue samples. This suggested that the detection of miR-183/FOXO1 in peripheral blood may have potential clinical values. In the future, we also need to study the biological function of miR-183 at cellular level, and further discover the direct evidence of the relationship between the regulation of FOXO1 by miR-183 gene and the disease.

To summarize, the present study demonstrates that the expression of FOXO1 in tumor tissues and blood from gastric cancer patients with *H. pylori* infection is significantly down-regulated, and may be related to the up-regulation of miR-183. *H. pylori* may regulate FOXO1 expression through miR-183 to affect the pathological process of gastric cancer. This study provides theoretical basis for the prevention, diagnosis and treatment of the disease.

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Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

*Author contributions.*CQ, CD and YL contributed to the design of the study. CQ, LL, JW and YJ performed the experiments. CQ, CD and LL analyzed the data. CQ and YL interpreted results and prepared the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethical approval and consent to participate. All procedures performed in the current study were approved by the Ethics Committee of Jinzhou Medical University. Written informed consent was obtained from all patients or their families.

Consent for publication. Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

Conflict of interest. The author declares that there is no financial or any conflict of interests related to this paper.

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