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Original Article

MiR-422a in gastric cancer cells directly targets CDC40 and modulates cell proliferation

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Abstract: MicroRNAs have been shown to be involved in a variety of different human cancers, including gastric cancer, functioning as post-transcriptional regulators of oncogenes or tumor suppressors. This study aimed to clarify the role of miR-422a in gastric cancer and further elucidate the pathogenesis thereof. To this end, miR-422a expression was initially determined in gastric cancer tissues and cells. Our results showed decreased miR-422a and increased cell division cycle 40 (CDC40) expression in gastric cancer. Dual-luciferase reporter assay further confirmed that miR-422a targeted CDC40. Altogether, this study showed that miR-422a downregulated CDC40, thereby affecting cell cycle progression. Moreover, restoration of miR-422a inhibited gastric cancer cell proliferation. In summary, this study has been the first to show that miR-422a was associated with CDC40 levels in human gastric cancer cells and that disease development may be attributed to CDC40.

Keywords: miR-422a, CDC40, gastric cancer, proliferation

Introduction

Over the past few decades, the incidence of gastric cancer has steadily decreased worldwide. However, gastric cancer remains one of the leading causes of cancer-related mortality globally, especially among countries in the Western Pacific Region, including Japan and China [1, 2]. A recent survey showed that approximately half of new gastric cancer cases worldwide have occurred in China, more than 90% of which have been found to be advanced [3]. Moreover, the molecular pathogenesis of gastric cancer still remains to be explored.

MicroRNAs (miRNAs) are a highly conserved group of small, noncoding, regulatory RNA molecules that are approximately 18-25 nucleotides in length [4-6]. The discovery of miRNAs in the early 1990s had opened a new era in understanding transcriptional and post-transcriptional regulation of gene expression in biological processes. Studies have shown that miRNAs regulate the expression of most human genes and modulate cell development, differentiation, proliferation, and apoptosis [7-9]. Hence, alterations in miRNA expression have been associated with various types of human diseases, such as cancer [10, 11]. Depending on their target, however, miRNAs may serve as a suppressor or promoter of tumorigenesis.

In recent years, functional and prognostic studies have proved that miRNAs play an important role in gastric cancer. Accordingly, Lei et al. reported that miR-145 acts as a tumor suppressor in gastric cancer that inhibits cell migration and metastasis by inhibiting MYO6 expression [12]. Recently, miR-188-5p has been identified as an oncogene in gastric cancer given its promotion of gastric cancer cell growth and metastasis [13]. Another study found that miR-193a-3p expression levels were significantly lower in gastric cancer compared to adjacent normal tissues and that ectopic miR-193a-3p expression inhibited gastric cancer cell growth and significantly suppressed cell invasion [14] Moreover, Shen et al. showed decreased miR-451 expression in gastric cancer tissues, while another study revealed that miR-1284 and miR-146a levels were deregulated in gastric cancer [15, 16].

In the present study, we found that miR-422a was significantly decreased in gastric cancer tissues, which could increase cell proliferation of gastric cancer cells in vitro. Our findings also confirmed that miR-422a targeted the cell division cycle 40 (CDC40) gene in gastric cancer cells. These results suggest that miR-422a may play an important role in the development of gastric cancer by modulating cell proliferation.

Materials and methods

Study approval

This study was conducted in accordance with all legal regulations including approval by Institutional Review Board of Zhejiang Provincial People's Hospital, Ethic Committees of Hangzhou Medical College, and the First People's Hospital of Anqing. Samples from patients with gastric cancer were collected after obtaining informed consent in accordance with the Institutional Review Board and the Declaration of Helsinki.

Cell lines and culture conditions

Human gastric cancer cell line SGC-7901, normal gastric epithelial cell GES-1, and 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin in a standard humidified incubator at 37°C in a 5% CO_2 and 95% O_2 atmosphere.

RNA extraction and RT-PCR analysis

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription reactions were carried out with 100 ng of total RNA using the TaqMan miRNA RT kit (Applied Biosystems) according to the manufacturer's instructions. Real-time polymerase chain reaction reactions were performed using a 7900 System (Applied Biosystems) using the Taq-Man miR-422a probe (Applied Biosystems) and gene-specific primers.

Integrated target prediction of miR-422a

We analyzed the potential miR-422a target genes with this database Targetscan7.2 (http://www.targetscan.org/vert_72/) and three other highly recognizable and promising miRNA-target prediction tools (MiRWalk 3.0, miRanda, and miRDB). The miR-422a target genes predicted by both four prediction programs were identified for further analysis.

In vitro transfection with synthetic microRNA

Synthetic miR-422a and a scrambled oligonucleotide were purchased from Dharmacon (Thermo Scientific). Briefly, 2×10^6 SGC-7901 cells were collected and electroporated with 0.25 μM oligonucleotide using Amaxa kit. Lipofectamine reagent (Invitrogen) was applied to transfect the mature miRNA precursor (pre-miR-422a, Applied Biosystems) in SGC-7901 cells. A random sequence pre-miR (pre-miR-Negative Control) (Applied Biosystems) was purchased and transfected using the above-mentioned protocol and used as a negative control (miR-NC).

Western blotting

Western blotting was carried out according to standard methods wherein protein lysates were prepared, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes, protein signals were visualized using the ECL by ChemiScope 3000 Exp system. Pre-stained Protein Ladder (Thermo) was loaded as a protein marker to estimate the molecular weight of the samples. Anti-CDC40 (1:500), Anti-eIF4E2 (1:500), Anti-BMP2 (1:500), Anti-BCL2L2 (1:1000), and Anti-β-Actin (1:1000) were purchased from Santa Cruz Biotechnology. Anti-β-actin served as an internal reference.

Proliferation assay

Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8) (Boshide) according to the manufacturer's protocol. The proportion to the number of living cells was determined at 460 nm using a microplate reader.

Cell cycle analysis by flow cytometry

The cell cycle was analyzed by quantifying DNA content using propidium iodide with flow cytometry. The transfected cells were collected at 72 and 120 h and then gently fixed in 70% ethanol overnight. Thereafter, the cells were treated with (500 U/mL) DNase-free RNase and stained

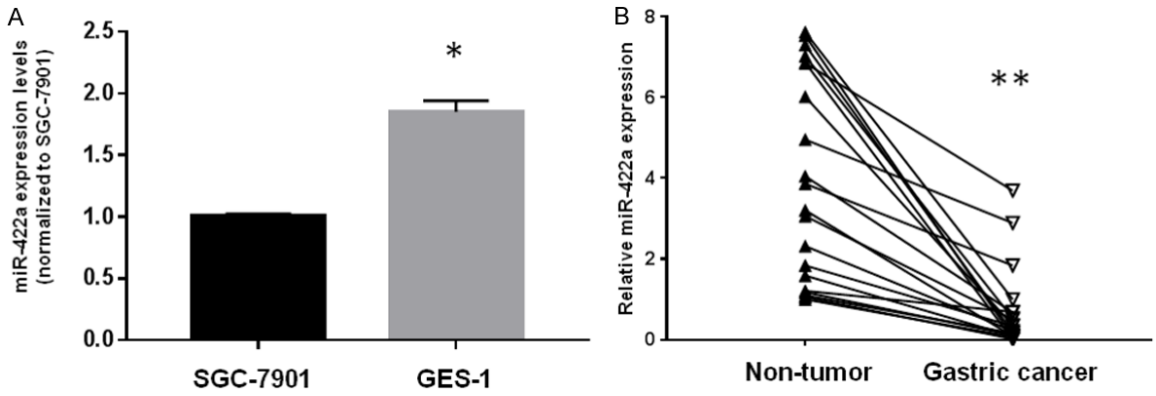

Figure 1. The expression of miR-422a in gastric cell lines and cancer tissues. MiR-422a expression was assessed using real-time PCR and was normalized using U6. A. Relative expression of miR-422a expression in gastric cancer cell line SGC-7901 compared with the non-malignant gastric epithelial cell line GES-1 cells (*$P < 0.05$). B. Relative miR-422a expression in 15 human gastric cancer tissues compared with matched adjacent normal gastric tissues (**$P < 0.01$).

with propidium iodide, and finally loaded on a FACSCalibur system to determine G0, G1, S, and G2 phase fractions.

Luciferase reporter assay

An empty luciferase reporter vector pEZX-MT01 was adopted to establish a wild-type CDC40 UTR luciferase reporter construct (pEZX-WT-UTR) and mutants (pEZX-Mut-UTR). Briefly, the 986-bp 3'-untranslated region (UTR) of CDC40 was cloned and ligated into pEZX-MT01. Then, mutations at the 7-mer seed region of the putative binding site of miR-422a in the 3'-UTR of CDC40 were designed. All constructs were verified through DNA sequencing. For the dual-luciferase assay, 293T cells were co-transfected with the reporter construct and pre-miR-422a or pre-miR-NC using Lipofectamine 2000. After 24 h, the transfected 293T cells were lysed and assayed for both firefly and Renilla luciferase using the Luc-Pair™ miR Luciferase Assay Kit (GeneCopoeia) according to the manufacturers' instructions.

Co-culture of SGC-7901 cells with Helicobacter pylori

The *H. pylori* strain was maintained in trypticase soy agar (with 5% sheep blood) and incubated at 37°C. An SGC-7901 cell/*H. pylori* co-culture model was established. Briefly, SGC-7901 cells were harvested and resuspended in fresh growth medium and seeded into a culture flask. After 48 h, *H. pylori* were added to the monolayer at a multiplicity of infection of 100 bacteria/cell and maintained in a 5% CO_2 humidified atmosphere at 37°C for another 24 h. After removing floating SGC-7901 cells, debris, and *H. pylori*, infected cells were then harvested for RNA extraction.

Statistics

Unless otherwise indicated, results were presented as mean ± standard error of the mean from at least three independent experiments. Statistical comparisons between groups were performed using the two-tailed Student's paired t-test with a p values < 0.05 being considered statistically significant.

Results

Reduced miR-422a expression in gastric cancer tissues

MiR-422a expression in SGC-7901 cells was compared to that in normal GES-1 cells. Accordingly, GES-1 cells had significantly higher miR-422a expression than SGC-7901 cells (1.7 fold; $P < 0.05$) (**Figure 1A**). Moreover, a significant difference in miR-422a expression between gastric tumors and adjacent tissues was confirmed ($P < 0.01$) (**Figure 1B**).

MiR-422a target genes in gastric cancer cells

Given the yet unknown function of miR-422a in gastric cancer, we were first interested in determining the target genes that miR-422a may

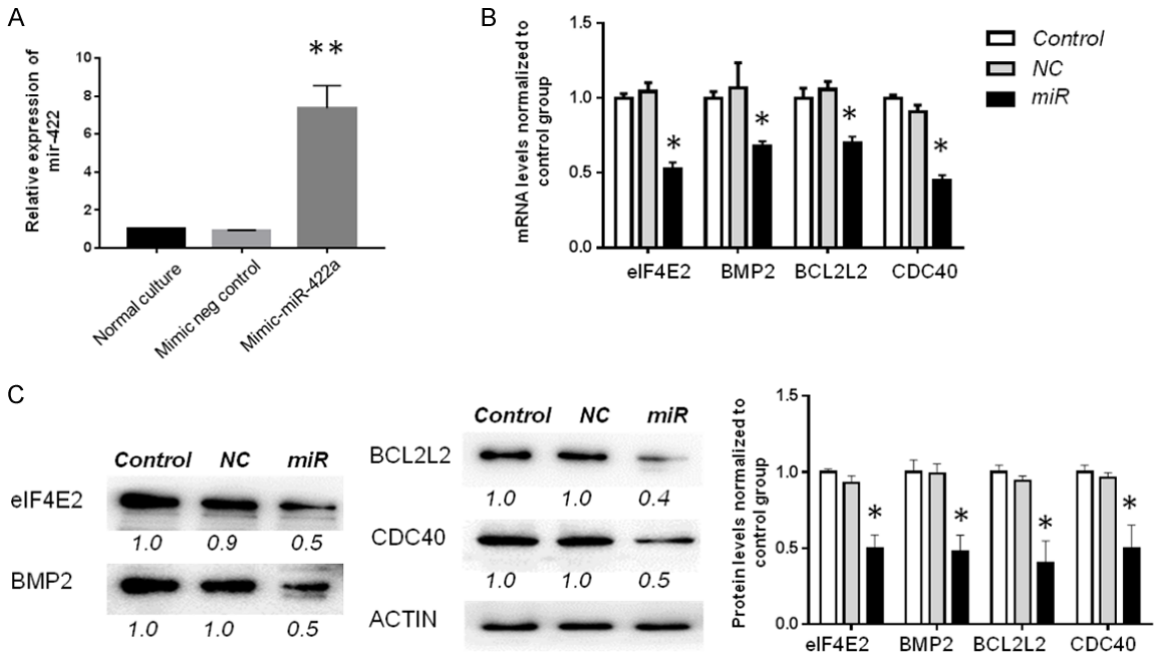

Figure 2. Transfection of synthetic miR-422a in gastric cancer cells. Mimic miR-422a or negative control oligonucleotide was transfected into SGC-7901 cells by electroporation. A. qRT-PCR showed that cells transfected with mimic had an approximately 6.3-fold increase of miR-422a compared with control (**P < 0.01). B, C. qRT-PCR and Western blots showed cells with mimic miR-422a transfection had low expression of miR-422a targets such as eIF4E2, BMP2, BCL2L2, and CDC40 (*P < 0.05; *P < 0.05).

regulate. Bioinformatics approaches predicted that miR-422a targeted Eukaryotic Translation Initiation Factor 4E Family Member 2 (eIF4E2), bone morphogenetic protein 2 (BMP2), Bcl-2-like protein 2 (BCL2L2), and CDC40. To further address this hypothesis, the aforementioned genes were validated in SGC-7901 cells upon miR-422a overexpression (P < 0.01) (**Figure 2A**). Accordingly, both quantitative RT-PCR (qRT-PCR) (P < 0.05) (**Figure 2B**) and Western blot analysis within 72 h of miR-422a overexpression showed significant downregulation of all four genes (P < 0.05) (**Figure 2C**). The same trend was observed in GES-1 cells (P < 0.01) (Supplementary Figure 1). Moreover, qRT-PCR was performed to determine differences in the expression of these four genes between gastric tumors and adjacent tissues. Interestingly, only CDC40 expression was found to have significantly differed between gastric tumors and adjacent tissues (P < 0.05) (**Figure 3**).

CDC40 as a direct target gene of miR-422a in gastric cancer cells

We further determined whether CDC40 was a direct target of miR-422a. Accordingly, transfection of the 3'-UTR CDC40 wild-type vector in gastric cancer cells with miR-422a overexpression resulted in the significant reduced luciferase activity, suggesting that CDC40 was a direct target of miR-422a (P < 0.05) (**Figure 4**). Moreover, mutations in the predicted seed region for miR-422a in the 3'-UTR of CDC40 abolished this effect, further reflecting that miR422a regulates CDC40 expression (P < 0.05) (**Figure 4**).

MiR-422a regulates cell cycle progression and proliferation

Next, we evaluated the role of miR-422a on gastric cancer cell cycle progression and proliferation. First, SGC-7901 cells were transfected with miR-422a and its corresponding control. The transfection efficiency and mRNA as well as protein levels of CDC40 were determined; both mRNA and protein levels of CDC40 were reduced after miR-422a overexpression (P < 0.01; P < 0.05) (**Figure 5A-C**). Further progression and proliferation assays showed that miR-422a overexpression significantly inhibited SGC-7901 cell proliferation by 42% at 48 h (P < 0.001) and 50% at 72 h (P < 0.001) (**Figure 5D**).

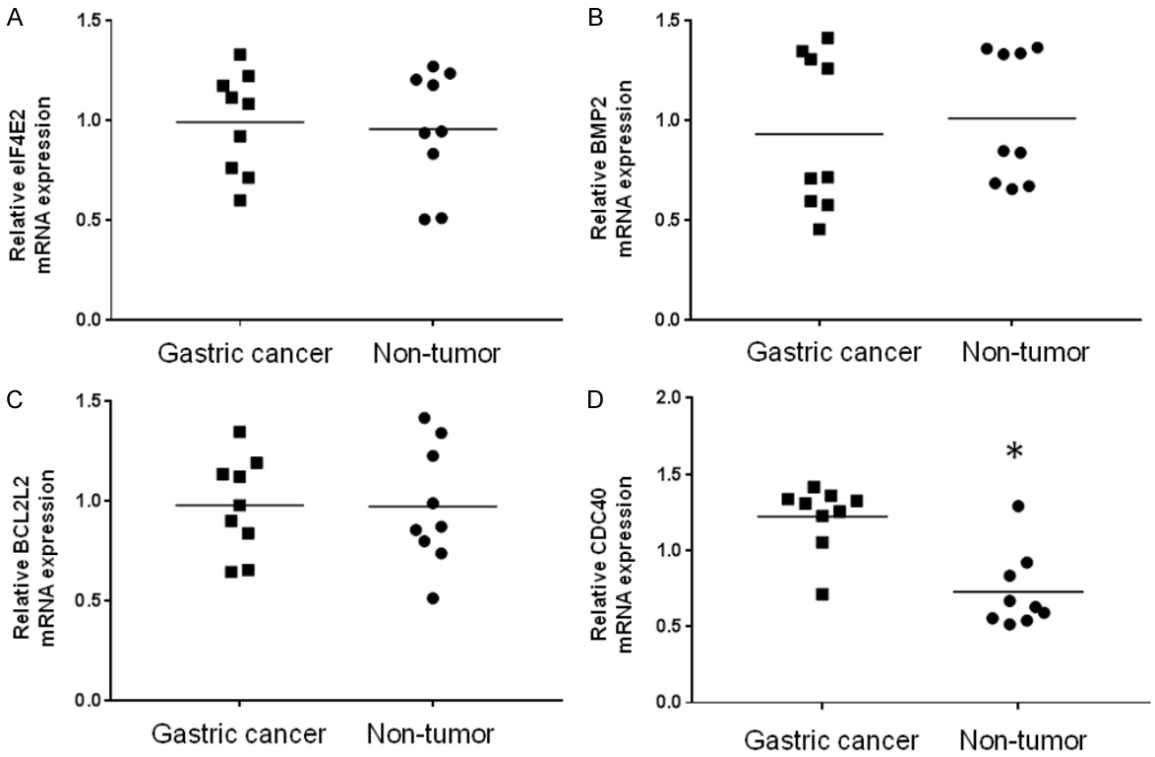

Figure 3. Identification of CDC40 as a potential target of miR-422a in gastric cancer. qRT-PCR was performed to evaluate miR-422a targets eIF4E2 (A), BMP2 (B), BCL2L2 (C), and CDC40 (D) in human gastric cancer tissues and compared with their matched normal gastric tissues. Significant differences in CDC40 expression but not eIF4E2, BMP2, or BCL2L2 expression (all $P>0.05$) were observed between gastric tumors and adjacent tissues (*$P < 0.05$).

Given the profound effect of miR-422a overexpression on cellular proliferation, we subsequently explored the mechanism for miR-422a-mediated growth inhibition. As such, flow cytometry assays were performed to determine the effect of miR-422a on cell cycle progression. Accordingly, miR-422a overexpression significantly increased the number of cells in the G2 phase while decreasing the number of cells in the S phase ($P < 0.001$) (**Figure 5E**). However, there was no significant difference in the number of apoptotic cells with and without miR-422a overexpression (data not shown). This indicates that miR-422a regulates cell cycle progression and proliferation, a result consistent with previous target prediction analyses.

Decreased miR-422a expression in SGC-7901 cells after H. pylori infection

Considering the prevalence of *H. pylori*-associated gastric cancer, we hypothesized that *H. pylori* infection may be associated with miR-422a expression. To test this hypothesis, SGC-7901 and GES-1 cells were infected with *H. pylori* for 24 h. As shown in **Figure 6**, both *H. pylori*-infected SGC-7901 and GES-1 cells showed lower miR-422a expression compared to parental controls ($P < 0.01$). As expected, CDC40 expression was increased after *pylori*-infection ($P < 0.01$) (Supplementary Figure 2).

Discussion

Although considerable advancements in treatment modalities have lowered mortality rates over recent decades, gastric cancer remains one of the most common malignancies, with approximately one million novel cases each year worldwide [1-3]. Previous studies have given much attention to miRNAs due to their importance in carcinogenesis and their potential for providing new diagnostic and treatment options for gastric cancer [7-11].

Human mature miR-422a is encoded by the gene MIR422A at 15q22.31 (64, 163, 129-64, 163, 218 bp) [17]. Some studies have determined the importance of miR-422a in human

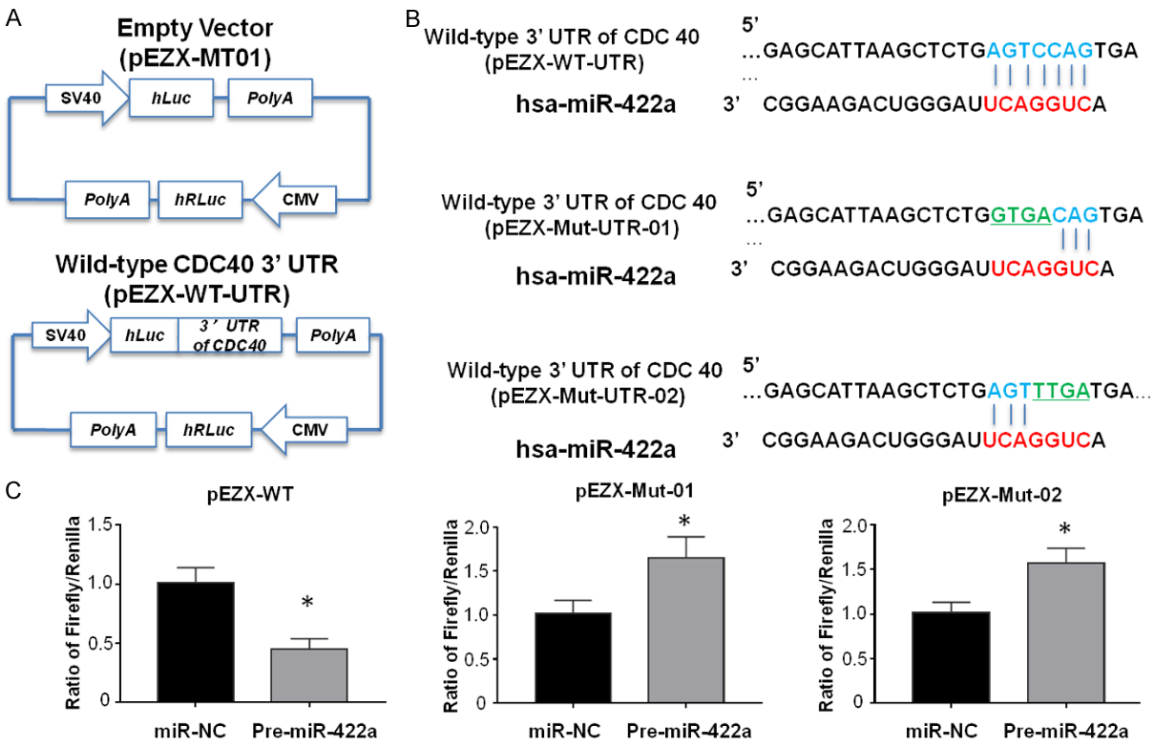

Figure 4. Effect of miR-422a on the 3'-untranslated region (UTR) of CDC40. A. Diagram of pEZX-WT-UTR vector with Renilla luciferase (hRLuc) as an internal control and firefly luciferase (hLuc) inserted at the upstream of the 3'-UTR construct. B. Diagram shows the putative binding site of miR-422a on CDC40 3'UTR along with mutations in the predicted seed region. C. Left figure shows the luciferase activity of pEZX-WT-UTR in SGC-7901 cells when co-transfected with pre-miR-422a or pre-miR-NC. Middle and right figures show the luciferase activity of pEZX-Mut-UTR-01 and pEZX-Mut-UTR-02 in SGC-7901 cells when co-transfected with pre-miR-422a or pre-miR-NC, respectively. All luciferase measurements were made in triplicate, while readings were performed 24 h post-transfection (*$P < 0.05$).

diseases. For instance, previous studies have demonstrated that miR-422a is commonly downregulated in various types of cancer [18, 19]. Moreover, colorectal tumor or laryngeal carcinoma tissues have been found to have lower miR-422a expression compared to normal tissues, while miR-422a has been proven to inhibit pathways that stimulate tumor cell proliferation in osteosarcoma [20, 21]. Thus, the present study sought to evaluate the role of miR-422a in gastric cancer.

Our results showed that gastric cancer cell SGC-7901 had lower miR-422a expression compared to normal gastric epithelial cell GES-1, with the results being confirmed through patients sample analysis. This suggests that miR-422a may be a potential biomarker for the diagnosis of gastric cancer. In addition, our study showed that differences in the expression of miR-422a lead to varying levels of cell proliferation. After searching miRNA target gene-predicting databases (TargetScan) and available published references, we identified four potential miR-422a target genes related to tumor growth, such as eIF4E2, BMP2, BCL2L2, and CDC40. eIF4E2 had been shown to participate in active translation in hypoxic mouse xenografts, while treatment with shRNA-targeting EIF4E2 was able to halt or reverse the growth of established tumors in mice [22, 23]. Moreover, data from *in vitro* and *in vivo* studies have suggested a pro-tumorigenesis role for BMP-2, while BCL2L2 overexpression, through amplification or other mechanisms, has been shown to promote the growth of a series of cancer cell lines [24-29]. However, qRT-PCR analyses of patient RNA samples obtained herein showed no significant difference in the expression levels of three potential target genes (eIF4E2, BMP2, and BCL2L2) between tumors and adjacent tissues. Although miR-422a could perhaps inhibit each of these target genes to some degree, none of the individual gene ex-

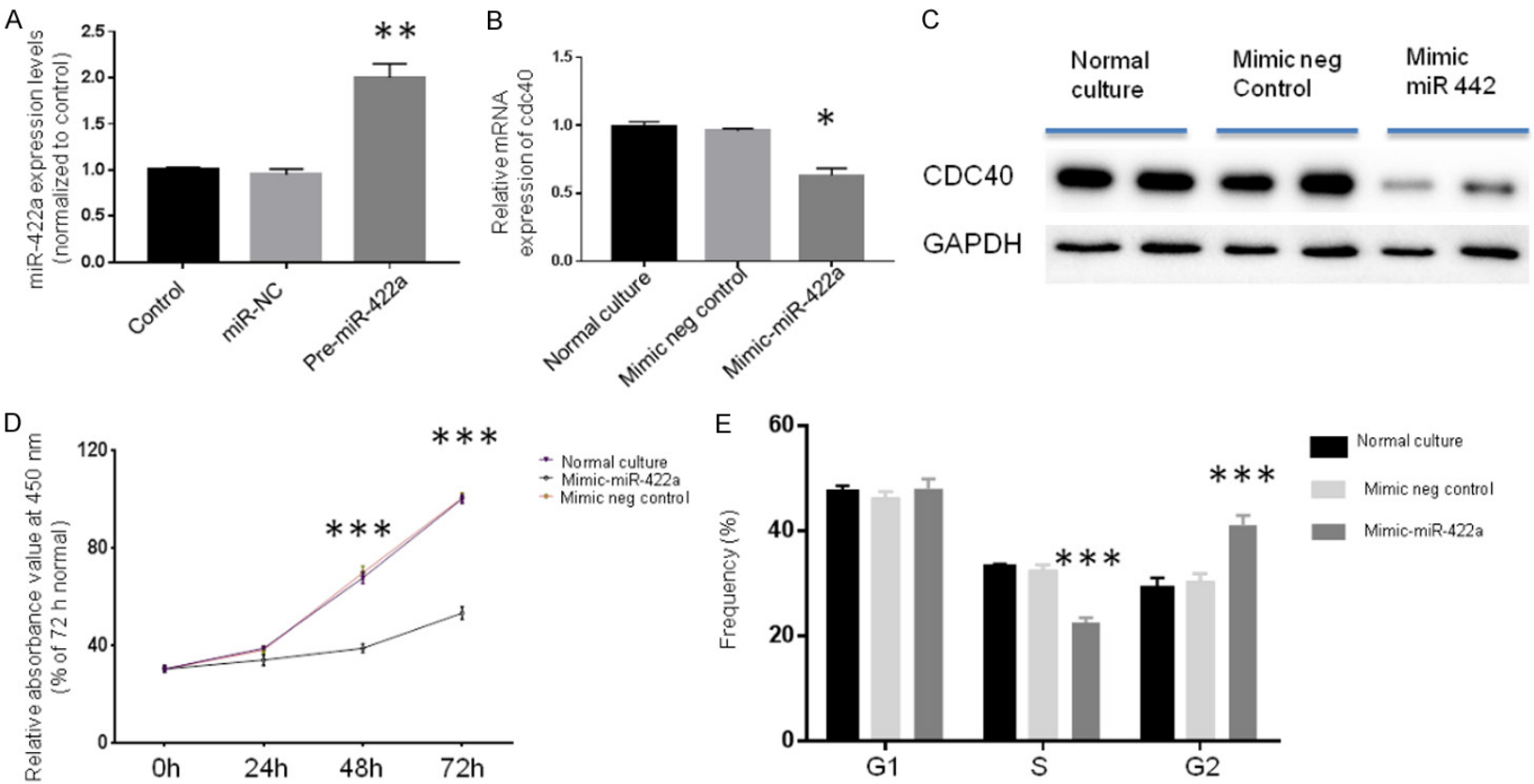

Figure 5. MiR-422a regulates cell cycle progression and proliferation. A. Ectopic expression of miR-422a in SGC-7901 cells was validated using qRT-PCR (**P < 0.01). B, C. Both mRNA and protein levels of CDC40 were reduced after miR-422a overexpression (*P < 0.05). D. Cell growth was inhibited in SGC-7901 cells with miR-422a mimic transfection (***P < 0.001). E. Flow cytometry analyses of cell cycle progression indicated that SGC-7901 cells were blocked in the G2/M phase after miR-422a overexpression (***P < 0.001).

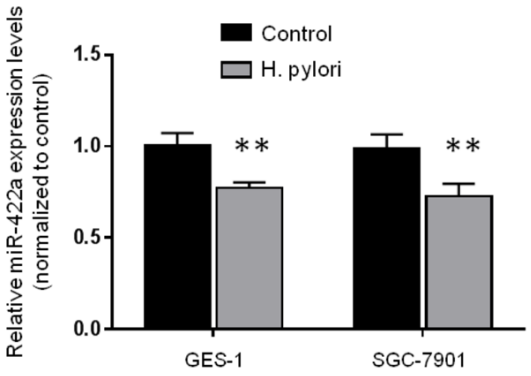

Figure 6. Down-regulation of miR-422a expression in gastric cancer cells infected with H. pylori. SGC-7901 and GES-1 cells were infected with *H. pylori* for 24 h. Data is shown as mean ± SD of three separate experiments (***P* < 0.01; ***P* < 0.01).

pressions differed significantly between tumors and adjacent tissues.

MiRNAs bind to the 3'-UTR of their target mRNA via at least 6- to 8-nucleotide-long complementary sequences [30]. They negatively regulate these target genes by cleaving the mRNAs or inhibiting their translation [31, 32]. In the present study, we showed that miR-422a could bind directly to the 3'-UTR of CDC40, while miR-422a overexpression downregulated CDC40 expression both at the mRNA and protein levels. These data indicate that miR-422a was associated with CDC40 levels in human gastric cancer. To the best of our knowledge, this has been the first study to investigate the function of miR-422a in gastric cancer.

In conclusion, the present study demonstrated that miR-422a is downregulated in gastric cancer cells. Furthermore, our results determined that miR-422a inhibited SGC-7901 cell proliferation through the suppression of CDC40. Therefore, miR-422a may be considered a potential biomarker and therapeutic target for gastric cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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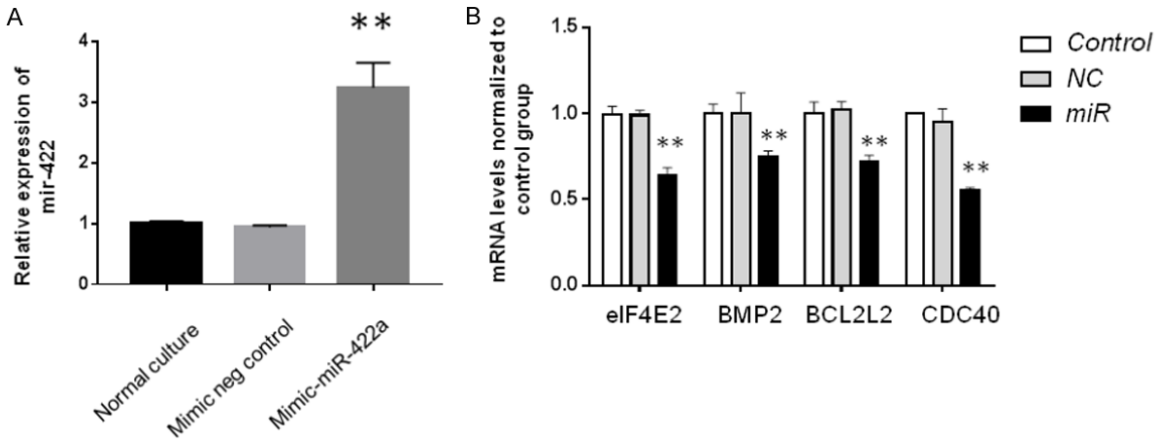
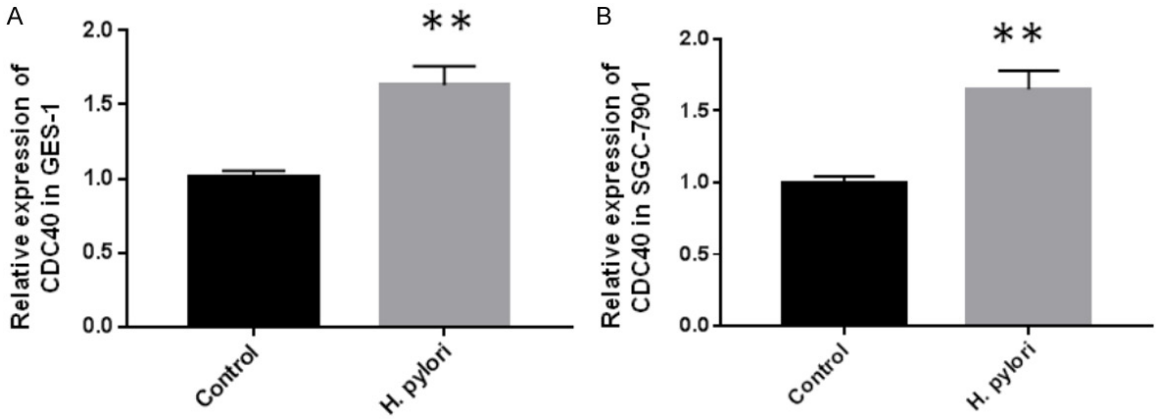


Supplementary Figure 1. Transfection of synthetic miR-422a in GES-1 cells. A. qRT-PCR showed that cells transfected with mimic had an approximately 3.2-fold increase of miR-422a compared with control (**$P < 0.01$). B. qRT-PCR showed cells with mimic miR-422a transfection had low expression of miR-422a targets, including eIF4E2, BMP2, BCL2L2, and CDC40 (**$P < 0.01$).



Supplementary Figure 2. CDC40 expression was increased in both GES-1 cells and SGC 7901 cells after pylori-infection. A. qRT-PCR showed that CDC40 expression was significantly increased in GES-1 cells (**$P < 0.01$). B. qRT-PCR showed that CDC40 expression was significantly increased in SGC 7901 cells (**$P < 0.01$).