Original Research Article

MiR-541-3p suppresses gastric cancer via negative regulation of HSF1

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Abstract

Purpose: *To explore the effects of miR-541-3P on the expression of heat shock transcription factor 1 (HSF1) in gastric cancer cells (GC).*
Methods: *The MicroRNA Target Prediction Database was used to predict whether miR-541-3p interacts with HSF1. Interaction was assessed by dual-luciferase reporter assays. Furthermore, miR-541-3p mRNA levels in GC cell lines were determined by qRT-PCR. Human GC cell lines MKN45 and NCI-N87 were transfected with miR-541-3p mimic. Cell apoptosis, proliferation, invasion, and migration were evaluated using flow cytometry, apoptosis assays, Edu assays, CCK-8 assays, and transwell assays, respectively. Caspase-3, Bcl-2, and cleaved caspase-3 expression levels were determined by western blot.*
Results: *Expression of miR-541-3p was significantly down-regulated in GC cells. Functionally, miR-541-3p mimic inhibited GC cell proliferation, migration, and invasion and induced apoptosis in vitro (p < 0.01). Mechanistically, miR-541-3p interacted with HSF1 and inhibited its expression. Overexpression of HSF1 counteracted the effects of miR-541-3p mimic in GC cells.*
Conclusion: *These results indicate that miR-541-3p suppresses the development of GC by targeting HSF1 and thus, is a possible strategy for for the management of GC.*

Keywords: *MiR-541-3p, Gastric cancer, Cell proliferation, Cell migration, Cell invasion, Apoptosis, Heat shock transcription factor 1*

INTRODUCTION

Gastric cancer (GC) is a digestive tract disease with high incidence that is often life threatening [1]. Due to a high mortality rate and frequent diagnosis at an advanced stage of disease, GC is the third most common cause of cancer deaths [2]. East Asia, Eastern Europe, and South America are current hotspots of GC incidence and mortality [3]. Gastric cancer is associated with various risk factors, such as *Helicobacter pylori* infection, cigarette smoking, and alcohol consumption [4]. The early stages of GC are often asymptomatic, and if symptoms are present at the time of diagnosis, the disease is often advanced and incurable [3]. Thus, it is important to find new treatments for GC and investigate the mechanisms of the disease.

Heat shock transcription factor 1 (HSF1) is a master regulator of the proteostasis network [5]. Mounting evidence suggests that HSF1 is elevated in various cancers, including breast, lung, colon cancer, hepatocellular and ovarian [6], and mediates cancer cell growth and migration [7]. However, it is not clear whether HSF1 interacts with microRNA (miRNA) in GC.

MiRNAs are abundant endogenous short-chain non-coding RNAs that contain 20 - 25 nucleotides. By pairing with target gene mRNA in the 3'-untranslated region (UTR), miRNAs regulate genes post-transcriptionally and can alter mRNA cleavage and destabilize target genes [8]. MiRNAs have been widely reported to participate in pathological processes [9]. Dysregulated miRNAs could play vital roles in carcinogenesis and tumor progression in human cancers including GC [10,11]. For example, miR-203 regulates cervical cancer cell cycle arrest and apoptosis by targeting Bmi-1 [12]. Thus, the miRNA/Gene network could provide novel insight into GC. MiR-541-3p has been shown to inhibit tumorigenesis in a variety of tumors, including prostate cancer [13] and hepatocellular carcinoma [14]. However, the functions and mechanisms of miR-541-3p in GC are unclear.

EXPERIMENTAL

Cell culture and transfection

Human GC cell lines (NCI-N87, AGS, SGC-7901, and MKN45) and the normal human gastric epithelial cell line (GES1) were purchased from the ATCC (Manassas, VA, USA). Cell lines were incubated in DMEM (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and propagated with 5% CO_2 at 37°C.

The MiR-541-3p mimic and inhibitor, mimic or inhibitor control, pcDNA3.1-HSF1 vector that overexpresses HSF1, and empty vectors were obtained from Ribobio, (Guangzhou, China). Lipofectamine 3000 (Thermo Fischer Scientific, Waltham, MA, USA) was used to transfect cell lines following the manufacturer's instructions.

Cell counting kit-8 (CCK-8) assay

Cell viability was measured with CCK-8 assays (Beyotime Biotechnology, Shanghai, China). Cells (1×10^4) were added to 96-well plates, and then 10 μL CCK-8 solution was added per well. The viabilities of MKN45 and NCI-N87 cells were measured at 24, 48, and 72 h in a microplate reader at 450 nm.

5-Ethynyl-2′-deoxyuridine (Edu) assay

The Edu assay kit (Beyotime Biotechnology) was used to detect cell proliferation. Cells were treated with Edu and then stained with 4′,6-diamid-ino-2-phenylindole (DAPI). Pictures of Edu-positive cells were taken using a fluorescence microscope (Nikon, Tokyo, Japan).

Transwell assay

Cells (3×10^4) were resuspended in medium without FBS in the upper well, and complete media with FBS was in the lower well. A transwell kit with an 8 μm pore size was used for migration assays. For invasion assays, upper chamber inserts with polyethylene terephthalate membranes (Millipore, Billerica, MA, USA) were used. After 48 h, in the cells upper chamber were discarded, while cells in the lower chamber were fixed, stained, and counted.

Flow cytometry

The Annexin V/Dead Cell Apoptosis Kit (BD Bioscience, San Diego, CA, USA) was used to detect apoptosis in MKN45 and NCI-N87 cells. In brief, cells were collected and resuspended, stained with annexin V and propidium iodide, incubated for 10 min in the dark, and analyzed using a flow cytometer (BD Bioscience).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Thermo Fischer Scientific). MiR-541-3p mRNA was reverse transcribed into complementary DNA (cDNA) by a microRNA Reverse Transcription Kit (Promega, Madison, WI, USA), and HSF1 mRNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). The SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism 7000 Sequence Detection System (Takara) and SYBR Green PCR kit (Takara) were used for qPCR. β-Actin was the reference gene for HSF1 mRNA, and U6 was the endogenous control for miR-541-3p mRNA.

Western blot assay

Cellular proteins were extracted using Radio Immunoprecipitation Assay buffer (Sigma Aldrich) and protease inhibitors. The BCA Protein Assay Kit (Beyotime Biotechnology) was used for protein quantification. Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride

membranes, which were then incubated with blocking buffer for 60 min. Then, the membranes were incubated with antibodies targeting Bcl-2 (1:800; Abcam, Cambridge, MA, USA), Caspase-3 (1:800; Abcam), Cleaved caspase-3 (1:800; Abcam), HSF1 (1:1000; Abcam), or β-actin (1:2000; Abcam) overnight at 4°C. The membranes were then incubated with secondary HRP-labeled rabbit IgG antibody (1:4000; Cell Signaling Technology, Danvers, MA, USA) for 60 min. The bands were detected using chemiluminescence.

Dual-luciferase reporter assay

The 3'-UTR of HSF1 containing mutant (mut) and wild-type (wt) putative binding sites for miR-541-3p was purchased from GenePharma and inserted into the psiCHECK-2 vector (Promega). MKN45 and NCI-N87 cells were co-transfected with HSF1-3'-UTR (wt or mut) reporter plasmids, miR-541-3p mimics, inhibitor, or negative control using Lipofectamine 3000. Luciferase activity was measured with a Dual Luciferase Assay Kit (Promega) 48 h after transfection.

Statistical analysis

All data in this paper are expressed as mean ± standard deviation (SD). Student's t-test was used for comparisons between two groups. One-way ANOVA was used to compare among more than two groups. Data were obtained from three independent experiments. Differences were considered to be significant at $p < 0.05$.

RESULTS

MiR-541-3p is down-regulated in GC cells and inhibits cell proliferation

Expression of miR-541-3p mRNA was significantly lower in GC cells than GES1 cells (Figure 1 A), and the MKN45 and NCI-N87 cell lines were selected for subsequent experiments. Transfection of miR-541-3p mimic significantly reduced cell viability in both MKN45 and NCI-N87 cells as shown by CCK-8 assays (Figure 1 B). Similarly, Edu assays showed that the miR-541-3p mimic significantly suppressed MKN45 and NCI-N87 cell proliferation (Figure 1 C). These findings suggest that downregulation of miR-541-3p represses GC cell proliferation.

MiR-541-3p mimic suppresses GC cell migration and invasion

Tumor metastasis is dependent on tumor cell invasion and migration. Transwell assays were performed to examine cell invasion and migration

after cells were transfected with miR-541-3p mimic. The results showed that miR-541-3p mimic reduced migration of GC cells, as well as invasion. These results demonstrate that miR-541-3p mimics can dramatically suppress GC cell migration and invasion (Figure 2).
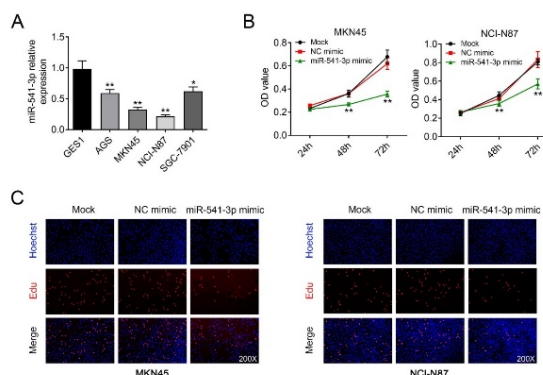

Figure 1: MiR-541-3p is down-regulated in GC cells and inhibits cell proliferation. (A) qRT-PCR analysis of miR-541-3p mRNA in GC cells treated with miR-541-3p mimic. (B) The CCK-8 assay was used to measure cell viability. (C) The Edu assay was used to measure cell proliferation. Data are presented as mean ± SD (n=3). *$p < 0.05$ vs. the indicated control group.
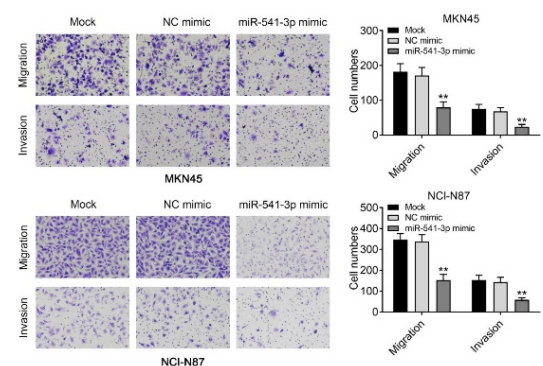

Figure 2: MiR-541-3p mimic suppresses GC cell migration and invasion. (A) MKN45 and NCI-N87 cell migration after transfection with miR-541-3p mimic, as measured by transwell assay. (B) MKN45 and NCI-N87 cell invasion after transfection with miR-541-3p mimic, as detected using transwell assay. Data are presented as mean ± SD (n=3). *$P < 0.05$ vs. the indicated group

MiR-541-3p mimic enhances GC cell apoptosis

To determine the effect of miR-541-3p mimic on GC cell apoptosis, flow cytometry was used to examine apoptosis, and western blotting was used to measure expression of cleaved caspase-3, Bcl-2, and caspase-3. Transfection of miR-541-3p mimic significantly increased cell apoptosis in MKN45 and NCI-N87 cells (Figure 3 A). Consistent with the above results, Bcl-2

protein levels were reduced in the miR-541-3p mimic group compared to mimic negative control, while cleaved caspase-3 levels were significantly increased (Figure 3 B). Thus, miR-541-3p promotes apoptosis in MKN45 and NCI-N87 cells.
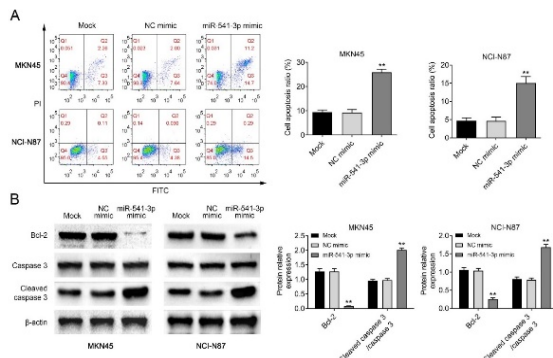

Figure 3: MiR-541-3p mimic enhances GC cell apoptosis. (A) Apoptosis in MKN45 and NCI-N87 cells was measured by flow cytometry following miR-541-3p mimic or mimic control transfection. (B) Protein expression of Bcl-2, caspase-3, and cleaved caspase-3 was detected by western blot in MKN45 and NCI-N87 cells. Data are presented as mean ± SD (n=3). *P < 0.05 vs. the indicated group

HSF1 is a direct target of miR-541-3p

To investigate the mechanism for the effects miR-541-3p on GC cells, potential binding sites in miR-541-3p were predicted using a bioinformatics analysis. Putative binding sites between HSF1 and miR-541-3p were identified (Figure 4 A). Dual-luciferase reporter assays showed that miR-541-3p mimic repressed the luciferase activity of the HSF1-wt 3'UTR, whereas miR-541-3p inhibitor increased the luciferase activity of the HSF1-wt 3'UTR (Figure 4 B). Moreover, miR-541-3p mimic inhibited HSF1 protein expression, while miR-541-3p inhibitor increased HSF1 protein expression (Figure 4 C). Taken together, these findings suggest that HSF1 is a target of miR-541-3p.

HSF1 mediates the regulation of miR-541-3p in GC

To determine the effect of HSF1 on miR-541-3p and GC cell viability, an HSF1 overexpression vector and miR-541-3p mimic were co-transfected into NCI-N87 cells. Overexpression of HSF1 reversed the reduction in cell viability induced by miR-541-3p (**Fig. 5A-B**). Elevated expression of HSF1 was also able to partially reverse the inhibition of cell migration and invasion by miR-541-3p (**Fig. 5C**). Similarly, increased expression of HSF1 counteracted the increased levels of apoptosis induced by miR-

541-3p (**Fig. 5D**), as well as reversing the effects of miR-541-3p on cleaved caspase-3 and Bcl-2 (**Fig. 5E**). Taken together, these results show that the effects of miR-541-3p on GC cell apoptosis, proliferation, invasion, and migration might be due to reduced expression of HSF1.
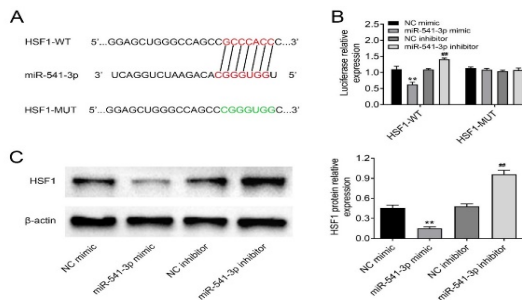

Figure 4: HSF1 is a direct target of miR-541-3p. (A) The sequence of a putative miR-541-3p binding site in HSF1-wt 3'UTR or HSF1-mut 3'UTR. (B) Luciferase activity in HSF1-wt 3'-UTR or its mutant form after treatment with miR-541-3p mimic or inhibitor. (C) HSF1 protein expression, as measured by western blot, after co-transfection with miR-541-3p mimic, inhibitor, or control into NCI-N87 cells. Data are presented as mean ± SD (n=3). *P < 0.05 vs. the indicated group; ##p < 0.01 vs. the mimic control group
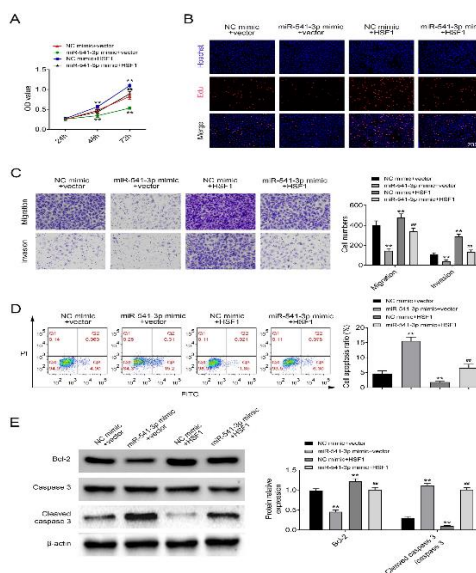

Figure 5: HSF1 mediates the effects of miR-541-3p on GC cells. (A, B) CCK-8 and Edu assays were used to examine cell viability and proliferation, respectively, in NCI-N87 cells transfected with miR-541-3p mimic, HSF1 overexpression vector, or both. (C) Tanswell assays were used to measure the cell migration and invasion. (D) Flow cytometry was used detect cell apoptosis. (E) Western blot was used to determine protein expression of Bcl-2, caspase-3, and cleaved caspase-3. Data are presented as mean ± SD (n=3). *p < 0.05 vs. the indicated group; ##p < 0.01 vs. the mimic control group

DISCUSSION

This study found that miR-541-3p mimic reduced protein levels of Bcl-2, cell proliferation, migration, and invasion and increased levels of cleaved caspase-3 and apoptosis, likely via interaction with HSF1 in MKN45 and NCI-N87 cells. Collectively, the results of this study indicate that miR-541-3p mimic decreased the progression of GC by targeting HSF1, suggesting a possible treatment for GC.

MiRNA participates in important physiological processes, including invasion, metastasis, cell apoptosis, differentiation, proliferation, and migration [15,16]. Studies have shown that miR-324-5p and miR-7 inhibited cell proliferation, while miR-7 also reduced cell invasion [17]. Hiroko *et al* investigated the role of miR-101-5p in tumor-suppression in breast cancer cells. They found that miR-101-5p is down-regulated in breast cancer and associated with a poor prognosis. Ectopic expression of miR-101-5p attenuated the observed phenotypes in breast cancer cells [18].

In another study, Deng *et al* discovered that miR-192 and miR-215 are up-regulated in GC tissues and associated with increased cell proliferation and migration [19]. Moreover, increasing evidence has suggested that miR-541-3p can act as an antioncogene in several types of tumors [20]. However, the effect of miR-541-3p on GC has not been studied. The present study showed that the miR-541-3p mRNA in GC cell lines was decreased. MiR-541-3p also inhibited cell proliferation, invasion, and migration and promoted apoptosis. These results suggest that miR-541-3p may act as antioncogene in the progression of GC and plays regulatory role in the occurrence, development, and metastasis of GC.

Mounting evidence has indicated that miRNAs regulate target gene expression in GC. For instance, miR-92b promotes GC cell proliferation by activating disabled homolog 2-ineracting protein [21]. However, miR-541-3p has not previously been found to regulate HSF1 expression. The results of dual-luciferase reporter assays indicated that miR-541-3p regulated HSF1 by binding to the 3'-UTR of HSF1, which altered the expression of HSF1. Specifically, HSF1 protein levels were significantly higher in cells treated with miR-541-3p inhibitor.

HSF1 is an important factor in tumorigenesis and is associated with a poor prognosis in GC patients [22]. Studies have shown that *HSF1* in human GC tissues is up-regulated and is associated with tumor progression [23]. In this study, overexpression of HSF1 reversed the repression of cell proliferation, invasion, and migration and elevation of apoptosis caused by the miR-541-3p mimic. These results suggest that miR-541-3p inhibits GC progression by targeting HSF1.

Bcl-2 regulates apoptosis in health and disease [24]. Caspase-3, encoded by the CASP3, is activated only in apoptotic cells. However, cleaved caspase-3 is active in both normal and apoptotic cells [25]. The protein levels of cleaved caspase-3, Bcl-2, and caspase-3 were determined by western blotting analysis. The results indicate that miR-541-3p mimic reduces Bcl-2 and increases cleaved caspase-3 protein levels, which results in apoptosis in GC cells.

CONCLUSION

MiR-541-3p plays a pivotal role in GC cells by binding HSF1, thus providing a new approach for improved treatment of GC.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zihan Zheng and Peng Zhou designed the study and supervised data collection; Yangyang Xiao analyzed and interpreted the data; and Qian Liu and Tao Wan prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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