miR-498 inhibits cell proliferation and metastasis by targeting FOXK1 in gastric cancer

Jinshan Liu
Qijiang Hospital of The First Affiliated Hospital of Chongqing Medical University
Ximing Huang
Qijiang Hospital of The First Affiliated Hospital of Chongqing Medical University
Liran Wu
Qijiang Hospital of The First Affiliated Hospital of Chongqing Medical University
Zhiqiang Zhao
Qijiang Hospital of The First Affiliated Hospital of Chongqing Medical University
Kun Fan (deyiliujinshan@163.com)
Qijiang Hospital of The First Affiliated Hospital of Chongqing Medical University https://orcid.org/0000-0002-4108-8083

Research

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Abstract

Objective: MiR-498 has emerged as a potential molecular target for several cancer. In this study, we aimed to investigate the important function and mechanisms of miR-498 in gastric cancer.

Methods: To detect the important roles of miR-498 in gastric cancer, we first measured its expression by RT-qPCR in gastric cancer cell lines. The impact of the miR-498 on gastric cancer cell proliferation were detected by CCK-8 and colony formation assays. The effect of the miR-498 on the cell apoptosis and cell cycle were detected by flow cytometry. We also used the scratch and transwell chamber assays to measure the cell migration and invasion. The expression levels of related proteins were assessed by western blot. The bioinformatics analysis was used to explore the target gene of miR-498. RT-qPCR and western blot assays were used to detect the expression levels of FOXK1 in response to miR-498 overexpression. In order to prove the role of FOXK1 in mediating the effect of miR-498 on the gastric cancer, CCK-8, colony formation, transwell chamber and flow cytometry assays were used for the further investigations.

Results: The expression level of miR-498 is downregulated in gastric cancer cell lines. Overexpression of miR-498 inhibited proliferation and migration/invasion, while promoted the apoptosis of gastric cancer cells. Bioinformatics analysis indicated that miR-498 targeted on FOXK1 to inhibit the gastric cancer *in vitro*.

Conclusion: MiR-498/FOXK1 axis may be a potential therapeutic target for the treatment of gastric cancers.

Introduction

Gastric cancer (GC) is the fifth most common malignancy with high morbidity and mortality in the world, while it is the third cause of cancer death after lung cancer and liver cancer. It was estimated in 2018, there were about over 1 million patents diagnosed with gastric cancer and the cases of death was over 780,000 [1]. With medical advanced, though the 5-year survival of gastric cancer patients is less than 30% [2].The main pathogenesis of gastric cancer is the infection of helicobacter pylori, the genetic susceptibility of host, the environmental factors, including the diet, smoking, and use of alcohol [3-4]. The tumorigenesis of gastric cancer is an extremely complicated process, which contains genetic and epigenetic changes of proto-oncogenes and cancer suppressors. Of note, dysregulated microRNAs (miRNAs) played an important role in gastric cancer [4]. For example, miRNA-21 may promote the growth of gastric cancer cells by adjusting and controlling PTEN/Akt signal passage mediated PEG2[5]. MiR-4317 represses the proliferation of gastric cancer cell by targeting and suppressing ZNF322[6]. MiRNA-194 is oncogenic and promotes GC cell proliferation and migration by activating Wnt/β-catenin signaling via suppression of SUFU[7].

MiRNAs are noncoding RNA molecules consist of 22 nucleotides, which could bind to a complimentary mRNA sequence, and thus resulting in post-translation repression or degradation and silencing. The miRNAs mediate the cell survival, proliferation, apoptosis, tumor growth, and metastasis by acting as a regulators of genes expression [8]. It has been widely reported that miR-498 were involved in the regulation of various cancers and were considered to be the prognostic biomarker and therapeutic target for prostate cancer [9], triple negative breast cancer [10], esophageal squamous cell carcinoma [11], liver cancer [12] and non-small cell lung cancer [13]. A previous study has found the overexpression miR-498 inhibits gastric cancer cell proliferation, migration and invasion and the long non-coding RNA UFC1 promotes gastric cancer progression by regulating miR-498/Lin28b [14]. However, the function of other miR-498's molecular targets in gastric cancer remain elusive.

The objective of our study was to investigate the roles and mechanism of miR-498 in gastric cancer. The present study outlined that miR-498 was downregulated in gastric cancer cell lines. Overexpression of miR-498 inhibited the gastric cancer cell proliferation, migration and invasion, while promoted the cell apoptosis. Notably, we identified a novel target, FOXK1, which mediated the inhibitory effect of miR-498 on the gastric cancer. Our findings highlight the potential therapeutic target in gastric cancer and are worthy for future investigation.

Materials And Methods

Cell lines and culture conditions

The human gastric epithelial cell line GES-1 and the gastric cancer cell lines AGS, MGC-803, SGC-7901 and SNU-1 were purchased from the Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All the cell lines were cultured with RPMI 1640 medium (Thermo Fisher Scientific, USA) supplemented 10% fetal bovine serum (Thermo Fisher, USA) and 1% penicillin-streptomycin (Gibco, USA). The cells were maintained in a humidified 37°C incubators (Thermo Fisher Scientific, USA)supplied with 5% of CO_2.

Cell transfection

NC mimic and hsa-miR-498 mimic were provided by Gene Pharma (Shanghai, China). Transfection of AGS and SGC-7901 cells were conducted using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, USA). Cells were cultured in 6-well plates for 48 h and were collected for subsequent analyses.

Lentivirus package

The lentiviral vector expressing FOXK1 was transfected into 293T packaging cells to obtain high levels of lentiviral particles in the culture supernatant. The AGS and SGC-7901 cells were cultured in 25-cm^2 dishes. Transfection was performed by adding polybrene (8 μg/ml) and 20 μl each viral dilution to the cells, thoroughly and gently mixing the solutions, and incubating the cells in 5% CO_2 at 37°C. After 18 h, the viral particles remaining in the supernatant were removed and the medium was replaced with fresh medium supplemented with 10% FBS (Thermo Fisher Scientific, USA.). The cells were incubated in 5% CO_2 at 37°C for an additional 72 h.

RNA extraction and RT-qPCR

Total RNAs were extracted from the treated cell lines GES-1, AGS, MGC-803, SGC-7901 and SNU-1 with TRIZOL reagent (Thermo Fisher Scientific, USA), and the cDNA were reverse-transcribed by Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). The reverse transcription reactions were: 37℃ for 15 min, 85℃ for 5 sec. RT-qPCR was conducted by SYBR-Green PCR Master Mix kit (Takara, Tokyo, Japan) and 7900 HT Fast system (Applied Biosystems, California, USA) with the following protocol: 94°C, 5 min; 40 cycles of amplification (94°C, 30 sec and 62°C, 40 sec); and then 72°C, 10 min. U6 were used as the internal control.

U6 upstream primer: 5'-CTCGCTTCGGCAGCACA-3', downstream primer:5'- AACGCTTCACGAATTTGCGT -3'.miR-498 upstream primer: 5'-GAGGGTTGGGTGGAGGCTCTCC-3', downstream primer: 5'-ATGACACGCAAATTCGTGAAGC-3'.

Cell counting kit-8 (CCK-8) assay

After transfection with NC mimic and miR-498 mimic, cells (2×10^3 cells/well) were seeded into 96-well plates and incubated in the humidified incubator at 37℃and 5% CO_2 for 0 h, 24 h, 48 h, and 72 h after inoculation. At each time point, 10 μl of the CCK-8 solution (Syngene, Nanjing, China) was added into each well, followed by incubation at 37℃ for another 2 h. The optical density value was detected at a 450 nm wavelength on a microplate reader (Thermo Fisher Scientific, USA)..

Colony formation assay

After transfection with miR-498 mimic, the cells were trypsinized, counted, seeded for colony formation assay in 6-well plates at the density of 100 cells per well. During colony growth, the culture medium was replaced every 3 days. The colony was counted only if contained more than 50 cells, and the number of colonies was counted the 6^{th} day after seeding. Colony formation rate was calculated with equation; colony formation rate =（number of colonies/ number of seeded cells）×100%. Each treatment was carried out in triplicate.

Flow cytometry analysis for apoptosis

AGS and SGC-7901 cell lines were transfected with either NC mimic or miR-498 mimic for 48 h. After that, the cells were washed, digested and transferred into centrifuge tubes and centrifuged at 1000g for 5 min for collection. 100 μl buffer solution were added to resuspend the cells to make sure the cell concentration was $1x10^6$. 5 μl Annexin V-FITC and 10 μl propidium iodide (PI) dye were further added into each tube for 10 min. Finally, the cell apoptosis was detected within 1h by Flow cytometer. The green fluorescence of FITC was detected in FL1 channel, while red fluorescence of PI was in FL2 or FL3 channel and then draw the two colors dot plot, the abscissa is FITC and the ordinate is PI.

Flow cytometry analysis for cell cycle

AGS and SGC-7901 cell lines were harvested, washed with 10 ml PBS with that centrifuged and resuspended to make sure the cell density at 1×10^6. The cells were fixed by 4.5 ml pre-chilled 70% ethanol (−20°C) and incubated at least 4 h at −20°C. After that, the cells were centrifuged for 3 min at 300 g and washed with 5 ml FACS buffer. Cells were stained with 0.5 ml Hoechst 33342 and Pyronin Y staining solution and were incubated at room temperature for 20 min and analyzed by flow cytometry. The flow cytometer was set up and adjusted with UV (355 nm) and blue (488 nm) lasers as well as proper filter sets. Doublets were excluded by creating a combination of same-channel bivariate plots utilizing Area *vs.* Height or Area *vs.* Width. Acquire the fluorescence and analyze cell cycle stages of each sample.

Western blot analysis

AGS and SGC-7901 cell lines transfected with NC mimic and miR-498 mimic were collected and then cracked in RIPA lysis buffer plus PMSF in low temperature. After extracted, the total protein concentration was detected by BCA assay kit (Santa Cruz, USA). Prepared protein samples were loaded in SDS-PAGE and then transferred into 0.22μm PVDF membranes and incubated with prepared antibodies, the primary antibody incubated overnight, and the secondary antibody for 2 h. Finally, enhanced chemiluminescence (Thermo Fisher Scientific, USA) visualized this membrane. The antibody β-actin was purchased from CST (Beverly, USA). Antibodies against the Cyclin D1, CDK2, P21, Cox-2, MMP-2, MMP-9 and FOXK1 were purchased from Abcam (Shanghai, China).

Scratch assay

Making 3 horizontal lines on the back of each hole of the 6-well plates and then culturing the AGS and SGC-7901 cells in the plates for a night after transfected with NC mimic and miR-498 mimic. When cells were grown to 90% confluence, creating a single wound in each hole perpendicular to the 3 horizontal lines and then washing the cells and culturing them in 37 °C 5% CO_2 incubator with medium without FBS. The images were digitally

photographed immediately after incubating for 0 h, 6 h, 12 h, 24 h. The original opening distances of the wound were set as 100%. The opening distances after 24 h were measured from three areas randomly selected per well, and the distances in three wells of each group were quantified and normalized by the original opening distance. The experiment was performed three times in triplicate, and the percentage of the migration rate was calculated by measuring the length of cell migration and expressed as a percentage compared to the control group. Migration rates = (treatment group cell migration distance/control group migration distance) × 100%.

Transwell chamber assay

Transwell chamber assay was used to detect the capacity for cell migration and invasion (8 μm diameters; Shanghai, China). AGS and SGC-7901 cells were collected and washed with PBS after transfected with NC mimic and miR-498 mimic for 48 h. Then resuspended the cells in DMEM without FBS. In total, 100 μl of suspension containing 5×10^4 cells was added into the upper chambers. The transwell chambers were placed into a 24-well plate that had already been covered with 500 μl of DMEM containing 10% FBS. 24-h later, non-migratory cells were gently removed, and the migratory cells were fixed with 100% methanol, stained with 0.5% crystal violet, washed with PBS, and imaged using an inverted microscope (Olympus, Tokyo, Japan). The migratory and invasive abilities were measured by counting respectively the migratory and invading cells.

Luciferase reporter assay

The 293T cell was transfected with NC mimic and miR-498 mimic to be assessed. After culturing the 293T for 48 h we firstly removed the growth medium and rinsed the cells with PBS and then added the cell culture lysis reagent to cover the cells (250 μl for a 60 mm dish), with that incubated the 293T for a short period. Finally, we extracted the cells with luciferase assay reagent (Promega, Shanghai, China) and measured the activity of promoters. The luciferase reporter was constructed by cloning 3′UTR region of the FOXK1 WT, FOXK1 Mut (mutant of functional miR-498 binding domain), whose sequences that bind to miR-498 were partly mutated in order to identify the binding specificity.

Statistical analyses

All experiments were repeated three times and the data are presented as the mean ± standard deviation (SD) using SPSS 18.0 (SPSS, Inc., USA). An unpaired *t*-test was performed to compare the difference of the two groups. At the meanwhile, a one-way ANOVA followed by Bonferroni multiple comparison test was used to detect differences between the two or more groups. $P < 0.05$ was considered as statistically significant.

Results

1. The expression level of miR-498 is downregulated in gastric cancer cell lines.

To detect the potential function of miR-498 in gastric cancer, we first measured its expression in gastric cancer cell lines. RT-qPCR analysis indicated that the expression level of miR-498 was decreased in gastric cancer cell lines, including AGS, MGS-803, SGC-7901, SNU-1, when compared with the normal gastric cell GES-1 **(Fig. 1A)**. Among these gastric cancer cells, the expression of miR-498 was lower expressed in the AGS and SGC-7901, thus we selected these cells for further investigation. To further identify the role of miR-498 in the gastric cancer progression, we overexpressed the miR-498 mimic into AGS and SGC-7901. As shown in **Fig. 1B**, RT-qPCR analysis revealed the miR-498 mimic upregulated in AGS and SGC-7901 cells.

2. Overexpression of miR-498 inhibits the proliferation and promotes the apoptosis of gastric cancer cells.

As shown in Fig. 2AB, the CCK-8 and colony formation assays revealed that miR-498 mimic inhibited the proliferation of AGS and SGC-7901. In order to explore the function of miR-498 on apoptosis, the flow cytometry assays were operated. After transfection with NC mimic and miR-498 mimic in AGS and SGC-7901, the miR-498 mimic increased the percentage of apoptosis of AGS and SGC-7901 cells **(Fig. 2C)**. Meanwhile, the cell cycle was analyzed by flow cytometry. We found that the miR-498 mimic upregulated the numbers of the AGS and SGC-7901 cells in G0/G1 phase, while decreased those cell numbers in phase S and G2/M **(Fig. 2D)**. At the molecular level, western blot assay were performed to examine the effect of miR-498 on cell cycle-related proteins, including Cyclin D1, CDK2 and P21. Compared to NC mimic, miR-498 mimic inhibits the protein expression levels of Cyclin D1 and CDK2, whereas promotes the protein expression levels of P21 in AGS and SGC-7901 cells **(Fig. 2E)**.

3. The overexpression level of miR-498 inhibits gastric cancer cells migration and invasion.

After transfection with NC mimic and miR-498 mimic, the migration and invasion of gastric cancer cells were demonstrated. The scratch assays indicated miR-498 significantly inhibited the migration of AGS and SGC-7901 cells **(Fig. 3A)**. The transwell chamber assays showed miR-498 overexpression inhibited the migration and invasion of AGS and SGC-7901 cells **(Fig. 3B)**. Western blot is also used to detect the expression level of migration and invasion-related proteins, such as Cox-2, which belongs to the cyclooxygenase (COX) enzymes family and has a crucial role in angiogenesis, invasion and immune suppression[18], as well as the MMP-2 and MMP-9, which are the members of matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases involved in the invasion or migration[19]. As shown in **Fig. 3C**, miR-498 mimic inhibited the expression of Cox-2, MMP-2 and MMP-9 both in AGS and SGC-7901 cells.

4. miR-498 targets on FOXK1.

MicroRNA targets are recognized through pairing between the miRNA seed region and complementary sites within target mRNAs [20]. By using the StarBase, the bioinformatics analysis indicated that FOXK1 possessed the potential binding sites of miR-498 **(Fig. 4A)**. Next, the luciferase reporter assay is carried out to investigate the effect of miR-498 on FOXK1 expression. The results indicated miR-498 mimic suppressed the luciferase activity of FOXK1 WT, whereas exhibited modest impact on the FOXK1 MUT in 293T cell lines **(Fig. 4B)**. Coincidence with these results, RT-qPCR and western blot analyses indicated that miR-498 mimic significantly inhibited the expression of FOXK1 in AGS and SGC-7901 cell lines **(Fig. 4C and D)**.

5. FOXK1 mediates the effect of miR-498 on the gastric cancer progression

After infection with the lentivirus expressing FOXK1, the RT-qPCR revealed the expression levels of FOXK1 were increased AGS and SGC-7901 cells **(Fig. 5A)**. CCK-8 and Colony formation assays indicated that miR-498 mimic inhibited the proliferation of AGS and SGC-7901 cell lines, while FOXK1 partially blocked such inhibition **(Fig. 5B and C)**. In addition, the apoptosis was detected by flow cytometry analysis. The results showed that miR-498 mimic promoted the apoptosis of AGS and SGC-7901 cell lines, while transfected with the FOXK1 expressing lentivector the promotion was antagonized **(Fig. 5D)**. Besides, the transwell chamber assays described miR-498 mimic inhibited the cell migration in gastric cancer cells, whereas the cell migration were increased in the miR-498 mimic combined the FOXK1 expressing lentivector group, when compared with miR-498 mimic combined with NC lentivector group **(Fig.5 E)**. All the results suggested that the FOXK1 is the target of miR-498, which mediates the inhibitory effect of miR-498 on the gastric cancer progression *in vitro*.

Discussion

Due to its high morbidity and mortality, gastric cancer raises the broad concern all over the world and is considered to be the deadly malignancy [21]. Patients diagnosed with early gastric cancer could be treated by surgery. For advanced gastric cancer, chemotherapy and targeted therapy are the main methods. Although many treatments are developed to cure gastric cancer, the recurrence, metastasis, and no response to chemotherapy, as well as the drug resistance, cause the unsatisfactory prognosis [22]. Except for the main helicobacter pylori, the incidence of gastric cancer is relevant to many factors. Among which, miRNA plays its role as potential biomarkers and therapeutic targets in gastric cancer. For instance, miRNA-21 promotes the growth of gastric cancer cells by adjusting and controlling PTEN/Akt signal passage via regulation of PEG2 [23]. miRNA-103a-3p promotes human gastric cancer cell proliferation by targeting and suppressing ATF7 *in vitro* [24]. MiR-136 promotes apoptosis in gastric cancer cells by targeting AEG-1 and BCL2[25]. The present study devoted to identify the roles of miR-498 on gastric cancer. Our results revealed that miR-498 acted as a repressor in gastric cancer cell proliferation, migration, invasion and apoptosis. The evidence emphasized the expression levels of miR-498 were lower in gastric cancer cell lines compared with the gastric epithelial cell line GES-1. The overexpression of miRNA-498 could inhibit proliferation, migration, invasion, and promotes apoptosis in AGS and SGC-7901 cells.

MiRNAs play essential roles by fine-tuning their target genes functionally associated with host genes [26]. In this study, we revealed FOXK1 is the target of miR-498. Forkhead box K1 (FOXK1) is a member of the FOX transcription factor family, which comprises diverse tissue-and cell type-specific transcription factors that are generally considered to be important regulators in the physiological development and the driving force for evolution. FOX proteins are multifaceted transcription factors involved in the development of the nervous system, kidneys, lungs, hair and immune systems. At the meanwhile, they are also associated with cancer. The FOX family promotes the development, maintenance, progression and metastasis of cancer at different regulatory levels through a highly complex and extensive network [27].

Previous works described FOXK1 played its important roles in proliferation, migration and invasion in several cancers [28]. For example, LINC01503 promotes colorectal cancer progression via acting as a competing endogenous RNA for miR-4492/FOXK1[29]. FOXK1 facilitates cell proliferation through regulating the expression of p21 and promotes metastasis in ovarian cancer [30]. MCM3AP-AS1 promoted growth and migration through modulating miR-138-5p/FOXK1 axis in pancreatic cancer, providing insights into MCM3AP-AS1/miR-138-5p/FOXK1 axis as novel candidates for pancreatic cancer therapy [31]. In gastric cancer, several studies demonstrated that FOXK1 was a target for some certain miRNAs (e.g. MiR-646, miR-4492, miR-137) which could inhibit the gastric cancer progression by mediating the cell proliferation, migration and invasion [32-34]. Given that the gastric cancer progression was correlated with the expression levels of miR-498 and FOXK1, our study also demonstrated that the miRNA-498 targets on FOXK1 and negatively regulates its expression in the gastric cancer cells. More importantly, overexpression of FOXK1 partially retarded the inhibitory effect of miR-498 on the gastric cancer cell proliferation and migration, thus providing a novel mechanism through which miR-498 regulates the gastric cancer progression and improving the posttranscriptional network between miRNAs and target genes in gastric cancer.

In conclusion, the present study demonstrated that miR-498 could inhibit the proliferation and migration/invasion, while promote the apoptosis of gastric cancer cells via the inhibition of FOXK1. Thus, miR-498/FOXK1 axis might be used as a diagnosed biomarker or therapeutic target for the gastric cancer and its associated diseases.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors agree to the publication of this manuscript.

Availability of data and material

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Conceived and designed the study: Jinshan Liu, Ximing Huang, Kun Fan. Performed the literature search and data extraction: Jinshan Liu, Liran Wu. Analyzed the data: Jinshan Liu, Ximing Huang, Zhiqiang Zhao. Drafted the manuscript: Jinshan Liu, Kun Fan.

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Figures

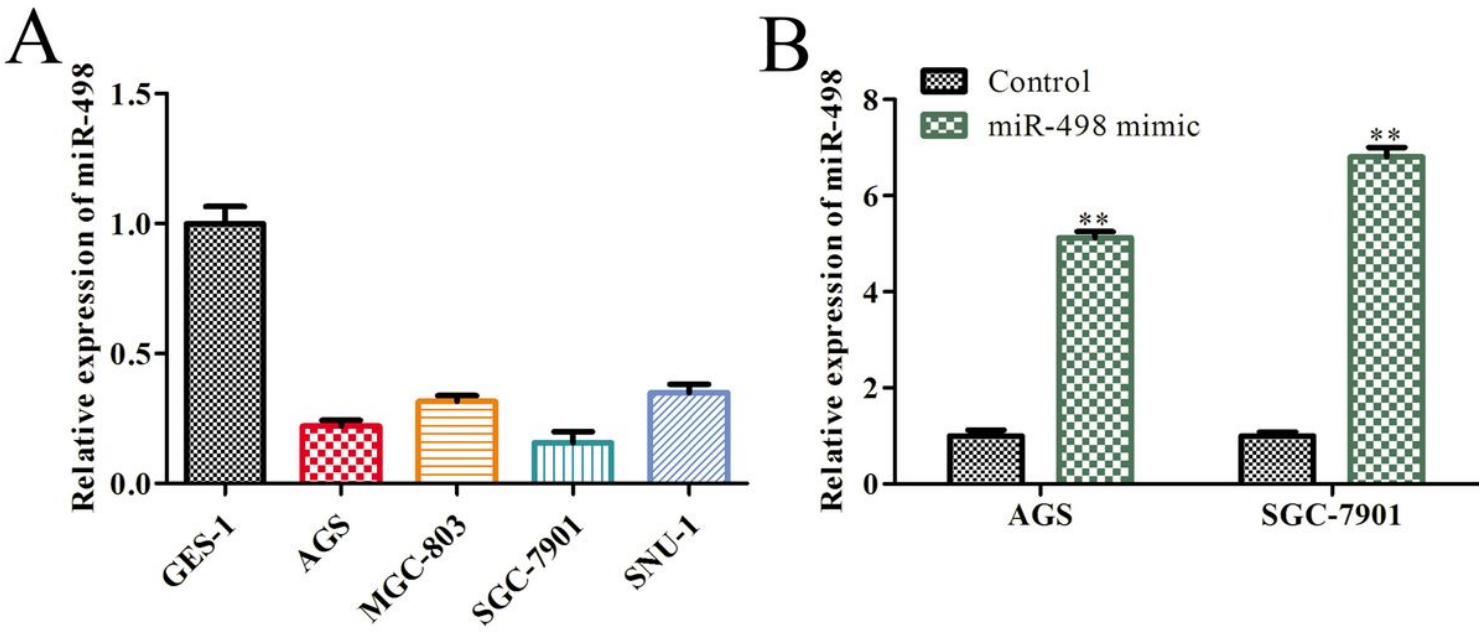

Figure 1

The expression level of miR-498 in gastric cancer cell lines. (A) The expression level of miR-498 in gastric epithelial cell line (GES-1) and gastric cancer cell lines (AGS, MGC-803, SGC-7901, SNU-1). (B) The expression level of miR-498 in AGS and SGC-7901 after transfected with NC mimic and miR-498 mimic. Asterisks indicated significant differences from the control (*P < 0.05, ** P<0.01, compared to GES-1group, #P < 0.05 and ## P < 0.01, compared to NC mimic group). All the data were presented as mean±SD.

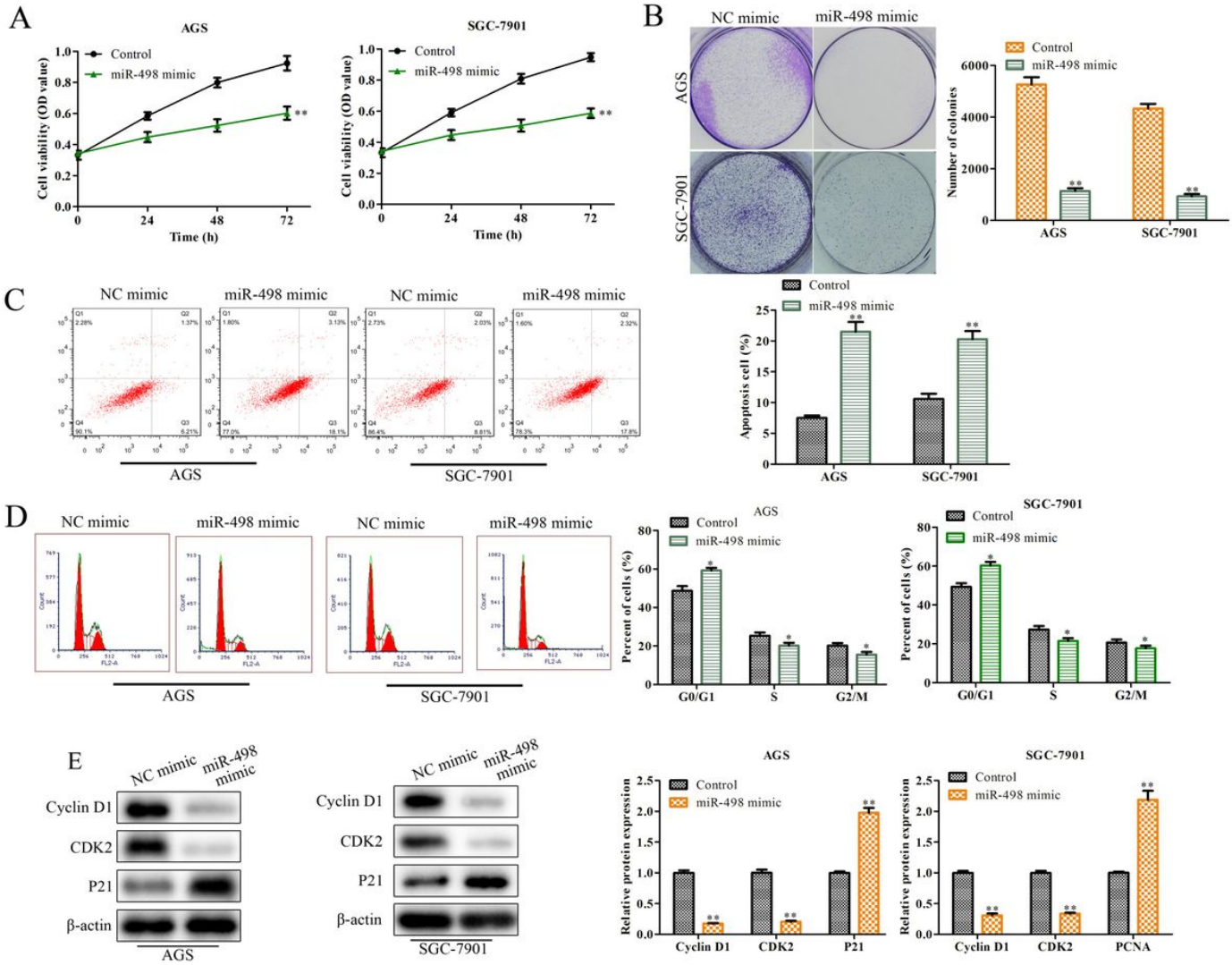

Figure 2

The effects of overexpression of miR-498 on apoptosis of gastric cancer cells. AGS and SGC-7901 cells were transfected with NC mimic and miR-498 mimic. (A) CCK-8 assay for proliferation after transfected with NC mimic and miR-498 mimic. (B) Colony formation assays for proliferation after transfected with NC mimic and miR-498 mimic.(C) Flow cytometry assay for apoptosis. (D) Flow cytometry assay for cell cycle. (E) Western blot assays for cell cycle-related proteins. Asterisks indicated significant differences from the control (* P < 0.05, ** P<0.01, compared to Control ,# P < 0.05, ## P<0.01, compared to NC mimic group).). All the data were presented as mean±SD.

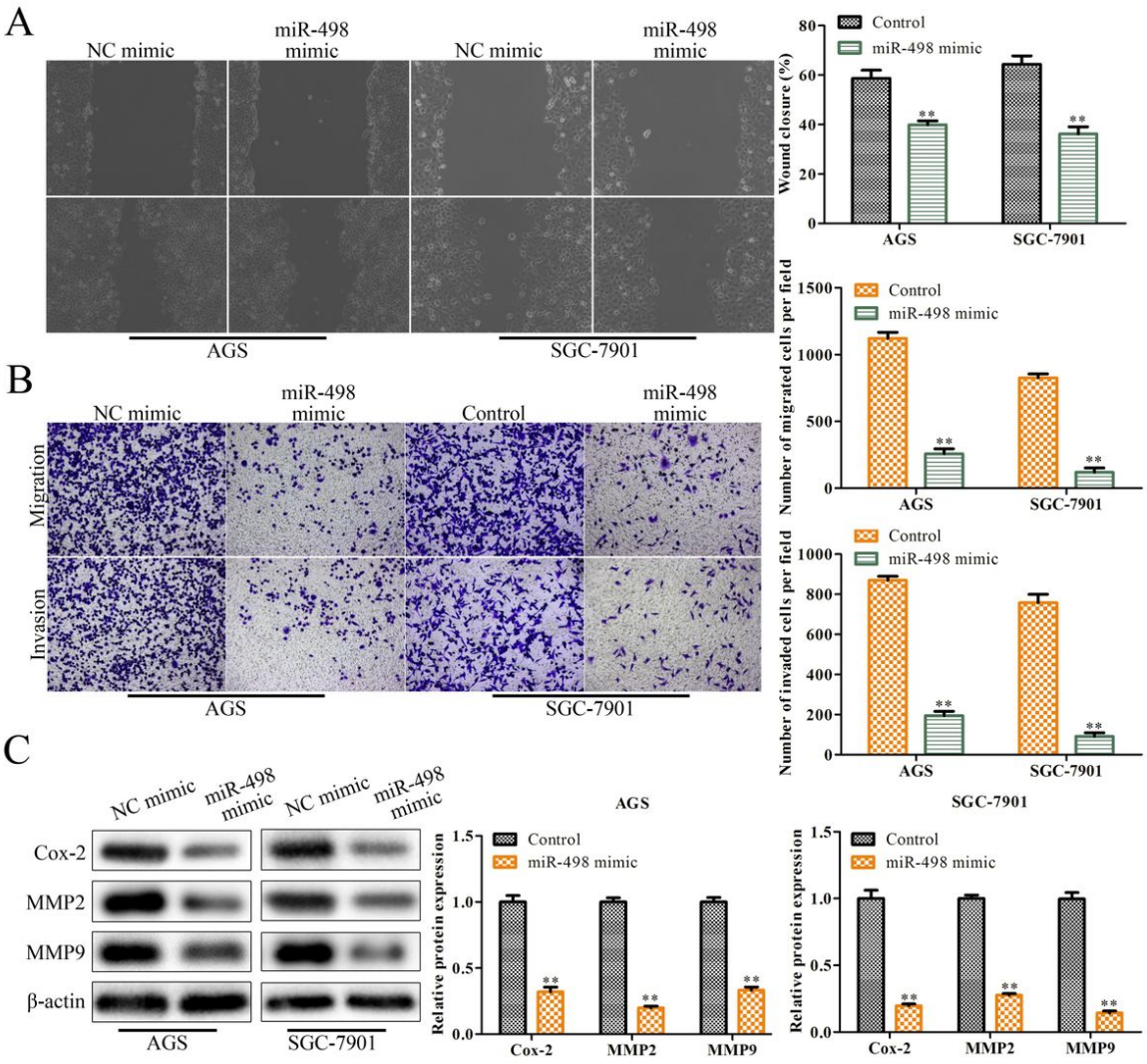

Figure 3

The effects of overexpression of miR-498 on migration and invasion in gastric cancer cells. AGS and SGC-7901 cells were transfected with NC mimic and miR-498 mimic. (A) Scratch assays for migration. (B) Transwell chamber assays for migration and invasion. (C) Western blot assays for migration and invasion related proteins. Asterisks indicated significant differences from the control (* P < 0.05, ** P<0.01, compared to NC mimic group). All the data were presented as mean±SD.

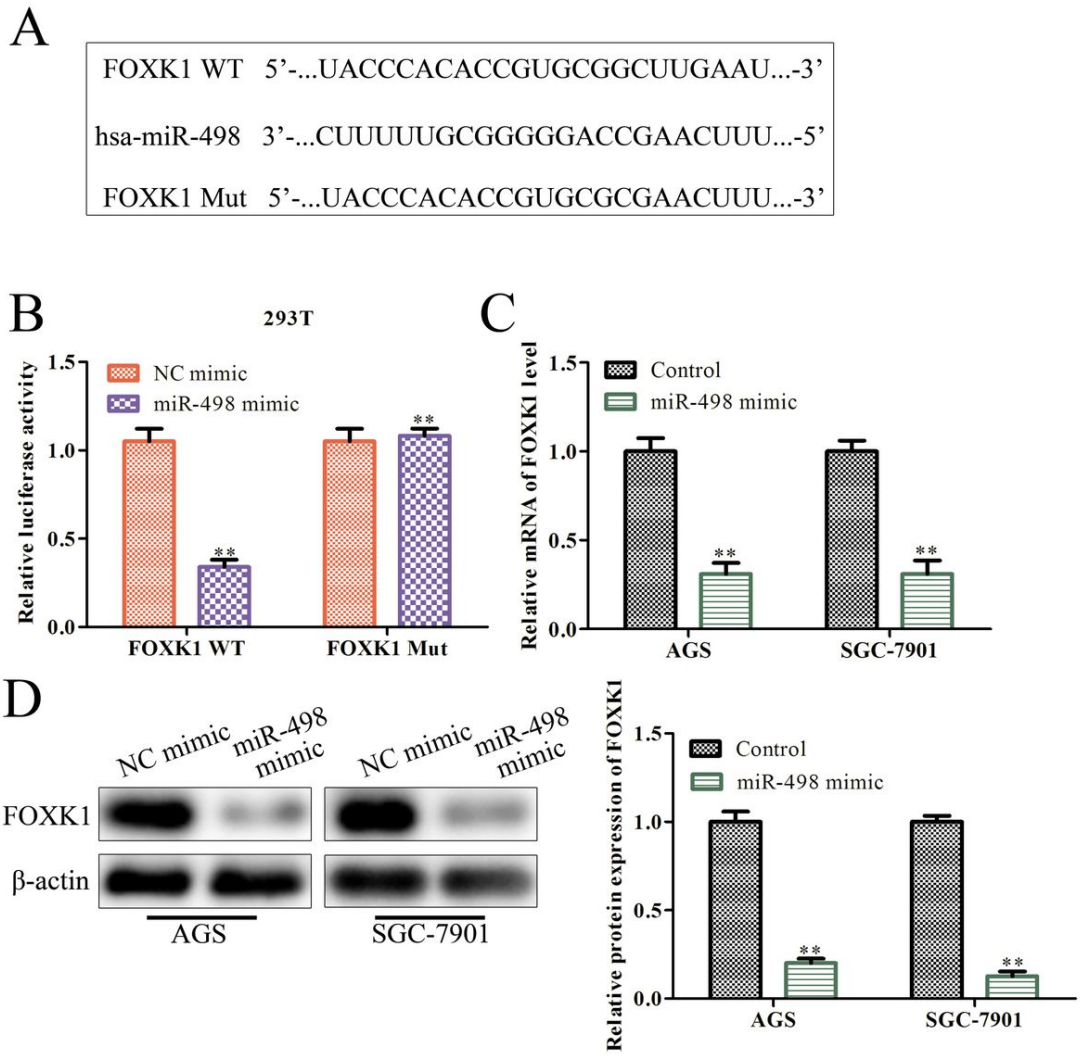

Figure 4

FOXK1 is a target gene of miR-498. AGS and SGC-7901 cells were transfected with NC mimic and miR-498 mimic. (A) The putative sequencing of 3'UTR FOXK1 for miR-498 binding. (B) Relative values of the luciferase signals. (C) The expression level of FOXK1 in AGS and SGC-7901 cells. (D) The protein expression level of FOXK1 in AGS and SGC-7901 cells. Asterisks indicated significant differences from the control (* $P < 0.05$, ** $P < 0.01$, compared to NC mimic group). All the data were presented as mean±SD.

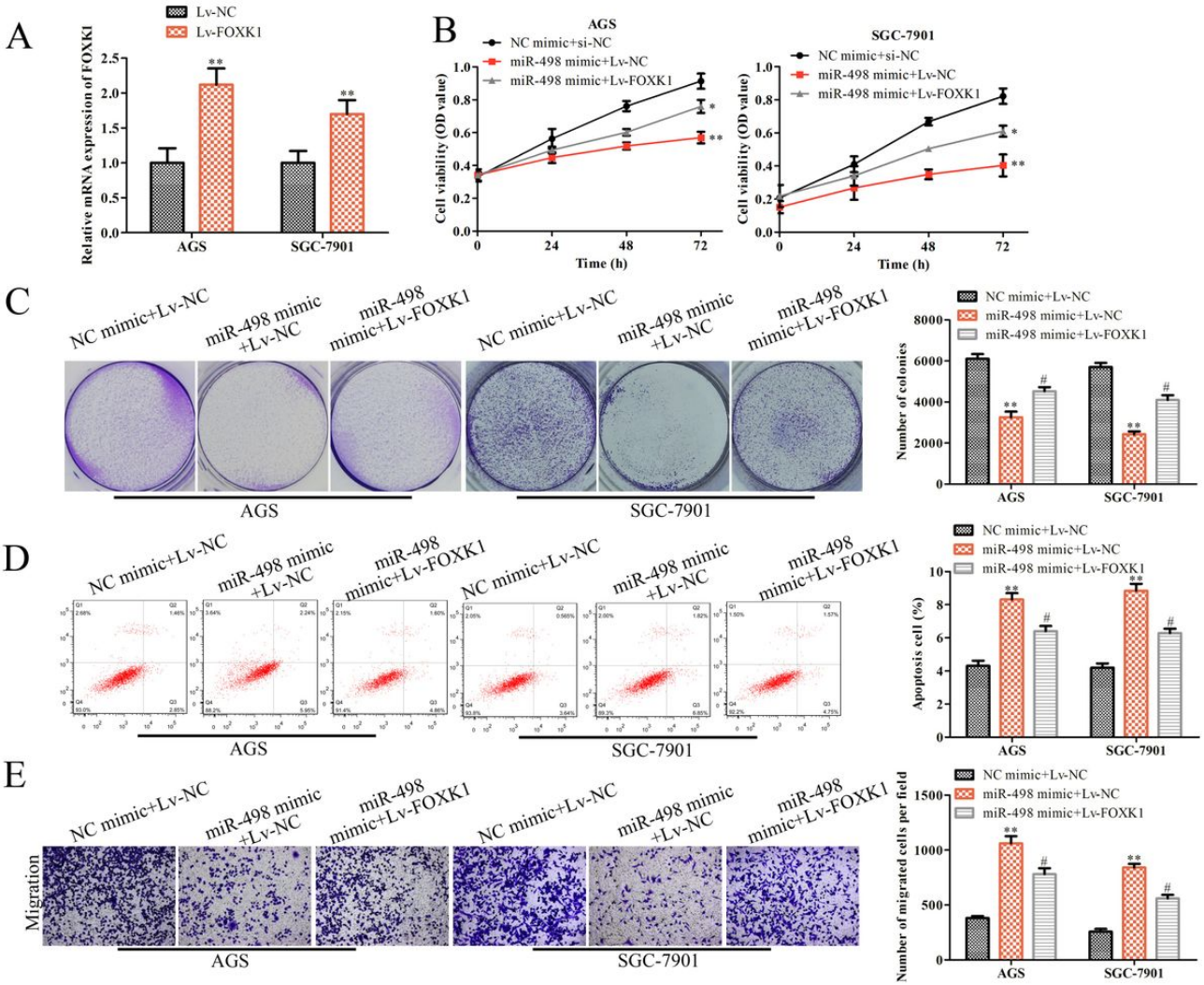

Figure 5

FOXK1 mediates the effect of miR-498 on gastric cancer progression. (A) RT-qPCR assays for the expression levels of FOXK1 in AGS and SGC-7901 cells after transfected with NC lentivector and FOXK1 expressing lentivector (**P< 0.01, compared to Lv-NC group). AGS and SGC-7901 cells were transfected with NC mimic combined with NC lentivector, miR-498 mimic combined with NC lentivector, miR-498 mimic combined with FOXK1 expressing lentivector. (B) CCK-8 assays for proliferation (C) Colony formation assays for proliferation. (D) Flow cytometry for the apoptosis. (E) Transwell chamber assays for migration. Asterisks indicated significant differences from the control. (* P < 0.05, ** P<0.01, compared to NC mimic + Lv-NC group). All the data were presented as mean±SD.