

DNMT3A-mediated down-regulation of microRNA-105 promotes gastric cancer cell proliferation

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Abstract. – **OBJECTIVE:** It has been well-established that microRNAs (miRNAs), a class of short non-coding RNA molecules, play an important role in the development of gastric cancer. In the present study, we focused on miR-105, a novel miRNA not previously linked to gastric cancer.

PATIENTS AND METHODS: 36 paired surgically resected gastric cancer tissues and matched adjacent normal tissues were used to detect the expression of miR-105. AGS cells were used to overexpress or silence of miR-105 and to determine its effect on several tumorigenic properties. A cell proliferation enzyme-linked immunosorbent assay was used to analyze the incorporation of BrdU during DNA synthesis of AGS cells. Total cDNA from AGS cells was used to amplify the 3'-UTR of YY1 by PCR and luciferase activity was determined using the Dual-Luciferase Reporter Assay System

RESULTS: We found that expression of miR-105 was reduced in gastric cancer tissues, compared with adjacent normal tissues, due to hypermethylation at its promoter region. Overexpression of miR-105 suppressed, whereas its inhibition promoted cell viability and proliferation. We further identified Yin Yang 1 (YY1) as a direct target of miR-105, by which miR-105 exerted its anti-proliferative role. Moreover, we found that DNMT3A was responsible for the down-regulation of miR-105 in gastric cancer cells.

CONCLUSIONS: Our data demonstrate that miR-105 inhibits gastric cancer cell proliferation and progression, which might provide a therapeutic target for cancer therapy.

Key Words:

Gastric cancer, microRNA, Cell proliferation, YY1.

Introduction

Gastric cancer has become one of the most common cancer and leading causes of cancer-induced mortality worldwide¹. Although great efforts have been made, its prognosis and the 5-year survival rate is relative low. Therefore, better understanding the pathogenesis and identification of new therapeutic targets are still important.

MicroRNAs (miRNAs) belong to a class of short and non-coding RNA molecules that inhibit target gene expression by binding to 3'-untranslated region, leading to degradation of messenger RNA and/or repression of translation^{2,3}. Increasing reports have shown that deregulation of several miRNAs plays a pivotal role in the development of gastric cancer⁴⁻⁸, by controlling cell proliferation, invasion, and apoptosis.

In the present work, we found that miR-105 was downregulated in gastric cancer tissues due to hypermethylation at its promoter region. Manipulating the expression of miR-105 could affect gastric cancer cell viability and proliferation. Furthermore, YY1 was identified as a direct target gene of miR-105.

Patients and Methods

Tissue Samples

36 paired surgically resected gastric cancer tissues and matched adjacent normal tissues were collected from patients during operation with informed consent. All tissue samples were

flash-frozen in liquid nitrogen immediately after collection and stored at -80°C until use. The study was approved by the First Affiliated Hospital of Soochow University Institutional Review Board.

Cell Culture

Gastric cancer cell lines (AGS and NCI-N87) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (EMD Millipore, Wanchai, Hong Kong, China). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 .

RNA Isolation and Real-time PCR

Total RNA from tissues or cell lines was harvested using Trizol according to the manufacturer's instructions (Invitrogen, Shanghai, China). Expression of mature miRNAs was assayed using TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) specific for miR-105. Small nuclear U6 snRNA was used as an internal control for normalization and quantification of miR-105 expression.

BrdU Assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime, Shanghai, China) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were performed in triplicate. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Western Blots

Cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 485-5p mM 2-ME, 2% w/v SDS, 10% glycerol). After centrifugation at $12000 \times g$ for 15 min at 4°C , proteins in the supernatants were quantified and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to NC membrane (Amersham Bioscience, Buckinghamshire, UK). After blocking with 10% nonfat milk in phosphate-buffered saline (PBS), membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) accord-

ing to manufacturer's instructions. Anti-YY1, p53 and GAPDH antibodies were purchased from Abcam (Cambridge, MA, USA).

Luciferase Reporter Assays

Total cDNA from AGS cells was used to amplify the 3'-UTR of YY1 by PCR. The YY1 3'-UTR was cloned into pMir-Report (Ambion, Foster City, CA, USA), yielding pMir-Report-YY1. Mutations were introduced in potential miR-105 binding sites using the QuikChange site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA). Cells were transfected with the pMir-Report vectors containing the 3'-UTR, and miR-105 mimics, negative controls for 36 hours. The pRL-TK vector (Promega, Madison, WI, USA) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM from at least three separate experiments. Differences between groups were analyzed using Student's *t*-test analysis. A value of $p < 0.05$ was considered statistically significant.

Results

Down-Regulation of miR-105 Expression in Gastric Cancer Tissues

The expression levels of miR-105 were detected in 36 cases of gastric cancer tissues and adjacent noncancerous tissues using a quantitative real-time PCR method. As a result, we found that its expression was significantly reduced in gastric cancer tissues in comparison with the adjacent normal tissues (Figure 1A).

It has been shown that epigenetic alterations, especially aberrant DNA methylation, play a critical role in gastric cancer development⁹. Therefore, to explore the mechanism for the down-regulation of miR-105, DNA methylation at the proximal promoter region of miR-105 was analyzed. Using methylated DNA immunoprecipitation (MeDIP) followed by quantitative PCR (MeDIP-qPCR), we found that the methylation in miR-105 promoter was up-regulated in gastric cancer tissues (Figure 1B). Besides, administration of 5-aza-cytidine (5-AZA), an inhibitor of DNA methylation, increased the expression

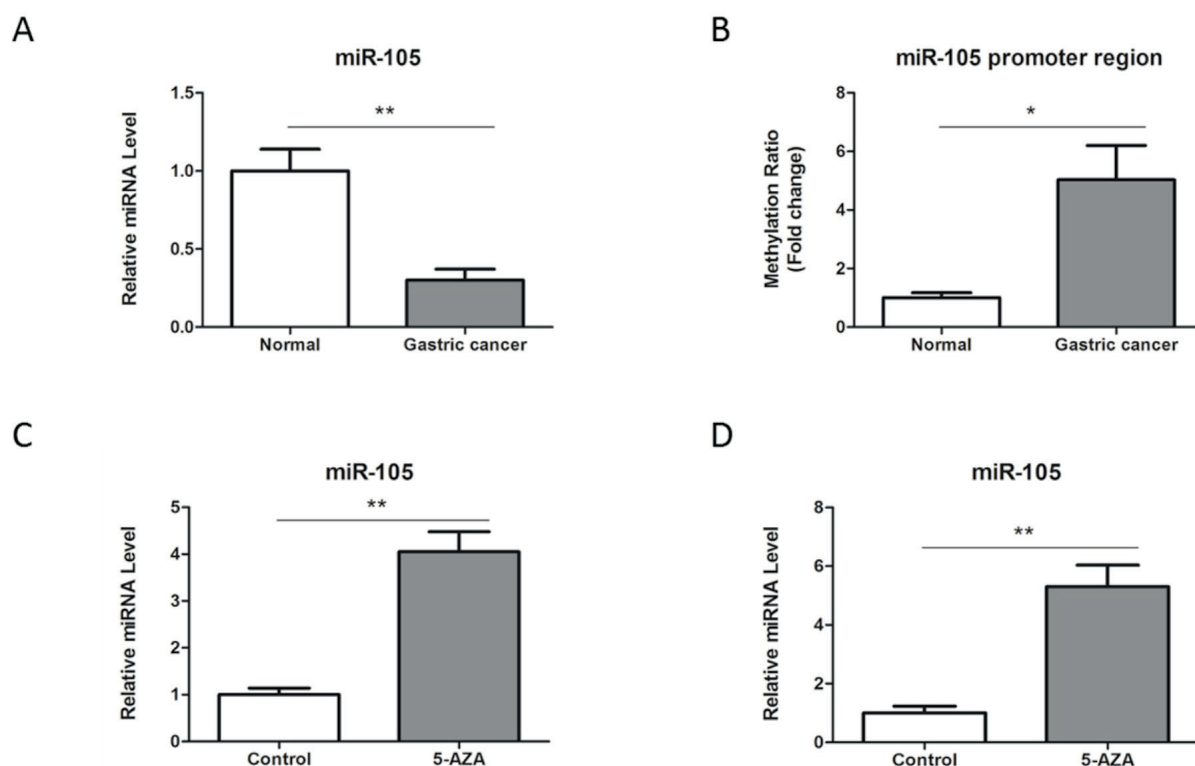


Figure 1. Up-regulation of miR-105 in gastric cancer tissues. **A**, Relative expression levels of miR-105 in gastric cancer and adjacent normal tissues. **B**, The ratio of methylated DNA levels in gastric cancer and adjacent normal tissues were measured by MeDIP-qPCR. **C-D**, Relative expression levels of miR-105 in AGS and NCI-N87 cells treated with 5'-AZA or vehicle control for 48 hr.

of miR-105 in AGS and NCI-N87 cells (Figure 1C-1D), suggesting that enhanced DNA methylation status in the miR-105 promoter contributes to its down-regulation in gastric cancer tissues.

DNMT3A Inhibits miR-105 Expression in Gastric Cancer Cells

Next, we verified which DNA methyltransferases (DNMTs) could regulate miR-105 expression. Therefore, vectors expressing three isoforms of DNMTs, DNMT1, DNMT3A and DNMT3B, were transfected into AGS cells. As a result, overexpression of DNMT3A, but not other two DNMTs, inhibited the expression of miR-105 (Figure 2A). Indeed, knockdown of DNMT3A resulted in an increased expression of miR-105 (Figure 2B-2C). In agreement, chromatin immunoprecipitation assays (ChIP) showed that the recruitment of DNMT3A at the promoter region of miR-105 was significantly up-regulated in gastric cancer tissues (Figure 2D), supporting the notion that miR-105 is negatively regulated by DNMT3A.

Roles of miR-105 in the Gastric Cell Proliferation

We then investigated the effects of miR-105 on cell proliferation and invasion in gastric cancer cells. miR-105 was markedly up-regulated in AGS cells following transfection with miR-105 mimics (Figure 3A). Abilities of cell proliferation and viabilities were significantly suppressed by miR-105 overexpression (Figure 3B-3C). Similar results were observed in NCI-N87 cells (Figure 3D-3F).

On the other hand, the transfection with miR-105 antisense, which inhibits its expression (Figure 4A and 4D), enhanced cell proliferation and viabilities in gastric cancer cells (Figure 4B-4C, 4E-4F), suggesting that miR-105 could act as a tumor suppressor in gastric cancer.

YY1 is a Direct Target of miR-105

The functions of microRNAs depend on its target genes. Therefore, the potential target gene of miR-105 was screened by miRWalk software¹⁰. Among these candidates, Ying Yang 1 (YY1), an important oncogene¹¹, was found to

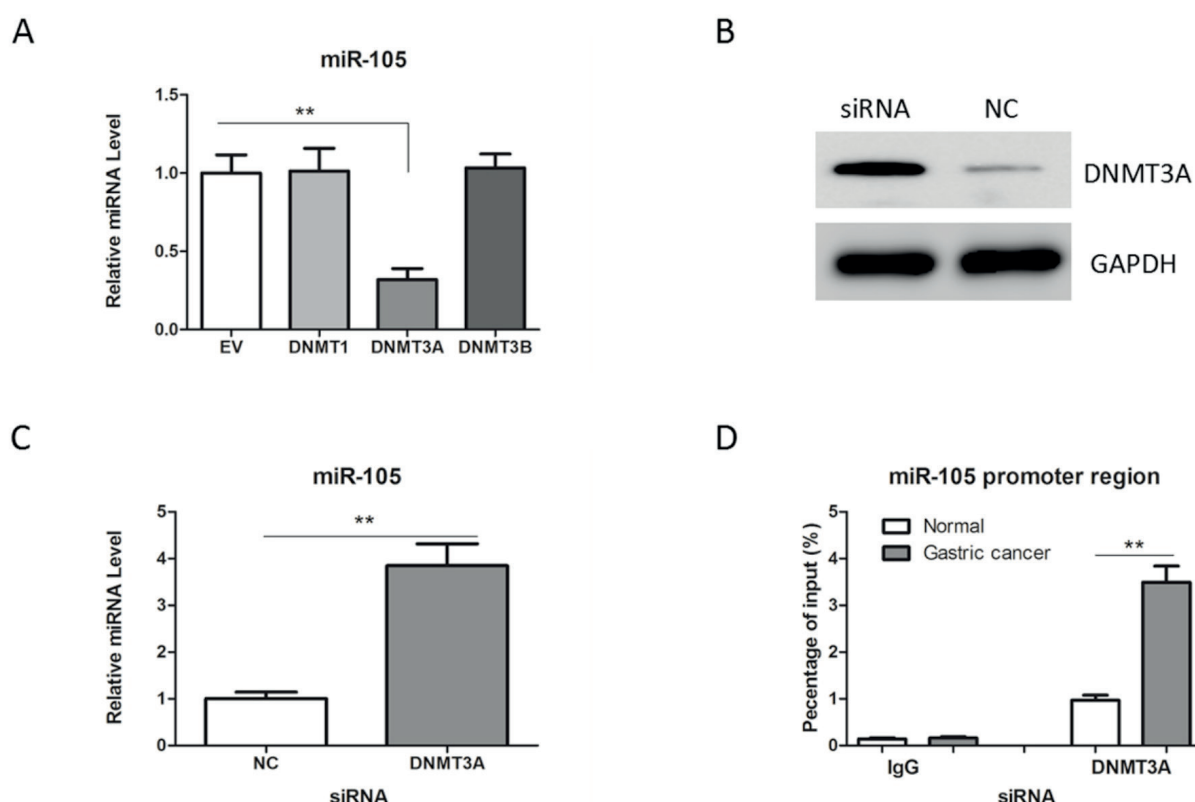


Figure 2. Regulation of miR-105 by DNMT3A. **A**, Relative expression levels of miR-105 in AGS cells transfected with plasmids expressing DNMT1, DNMT3A, DNMT3B or empty vector (EV). **B**, Representative protein levels of DNMT3A in AGS cells transfected with siRNA oligos targeting DNMT3A or negative control (NC). **C**, Relative expression levels of miR-105 in AGS cells transfected with siRNA oligos targeting DNMT3A or negative control (NC). **D**, Chromatin immunoprecipitation (ChIP)-qPCR analysis for recruitment of DNMT3A to the miR-105 promoter.

harbor a miR-105 binding site and selected for analysis (Data not shown). To explore whether miR-105 could regulate YY1 expression, the wild-type or mutant 3'-UTR region of YY1 was constructed into luciferase reporter plasmid. As shown in Figure 5A, overexpression of miR-105 mimics suppressed the wild-type luciferase activity. However, mutation of the miR-105 binding site significantly abolished the inhibitory effect of miR-105 (Figure 5A). Besides, endogenous YY1 expression was analyzed in gastric cancer cells with miR-105 overexpression or knockdown. Our Western blots showed that protein levels of YY1 were substantially down-regulated by miR-105 mimics (Figure 5B). In contrast, transfection of miR-105 antisense increased the protein levels of YY1 in gastric cancer cells (Figure 5C).

miR-105 Regulates Protein levels of p53

Previous studies^{12,13} have demonstrated that YY1 promotes cell proliferation through multi-

ple mechanisms including destabilizing p53 and stabilizing HIF-1. Here, the protein levels of p53 were detected. As expected, overexpression of miR-105 mimics increased, while its knockdown reduced protein contents of p53 (Figure 6A-6B). Therefore, we can conclude that reduced expression of miR-105 in gastric cancer up-regulated YY1, which affects its down-stream target genes, such as p53, to accelerate tumorigenesis.

Discussion

The roles of miRNAs in the development of gastric cancer have attracted recent attention by a variety of studies. The current work showed that miR-105 expression is significantly reduced in gastric cancer, compared with matching adjacent nontumoral tissue. Besides, we found that the methylation in miR-105 promoter was up-regulated in gastric cancer tissues and the DNA methyltransferase DNMT3A, but not other

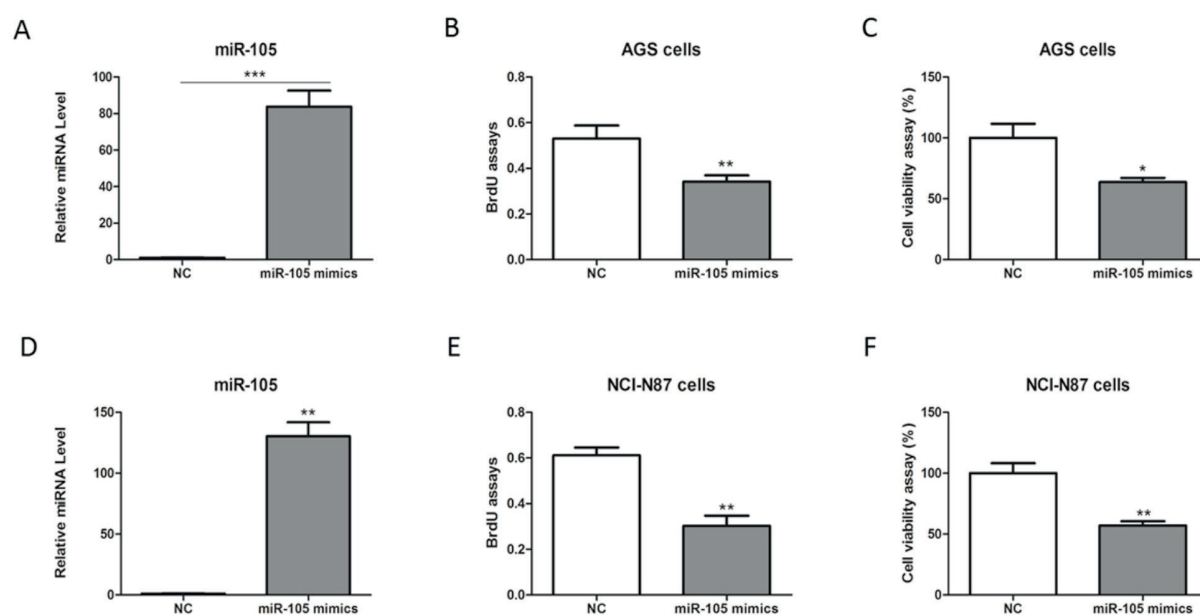


Figure 3. miR-105 inhibits cell proliferation. **A**, Relative expression levels of miR-105 in AGS cells transfected with miR-105 mimics or negative control (NC). **B-C**, Cell proliferation and viabilities in AGS cells transfected with miR-105 mimics or negative control (NC). **D**, Relative expression levels of miR-105 in NCI-N87 cells transfected with miR-105 mimics or negative control (NC). **E-F**, Cell proliferation and viabilities in NCI-N87 cells transfected with miR-105 mimics or negative control (NC).

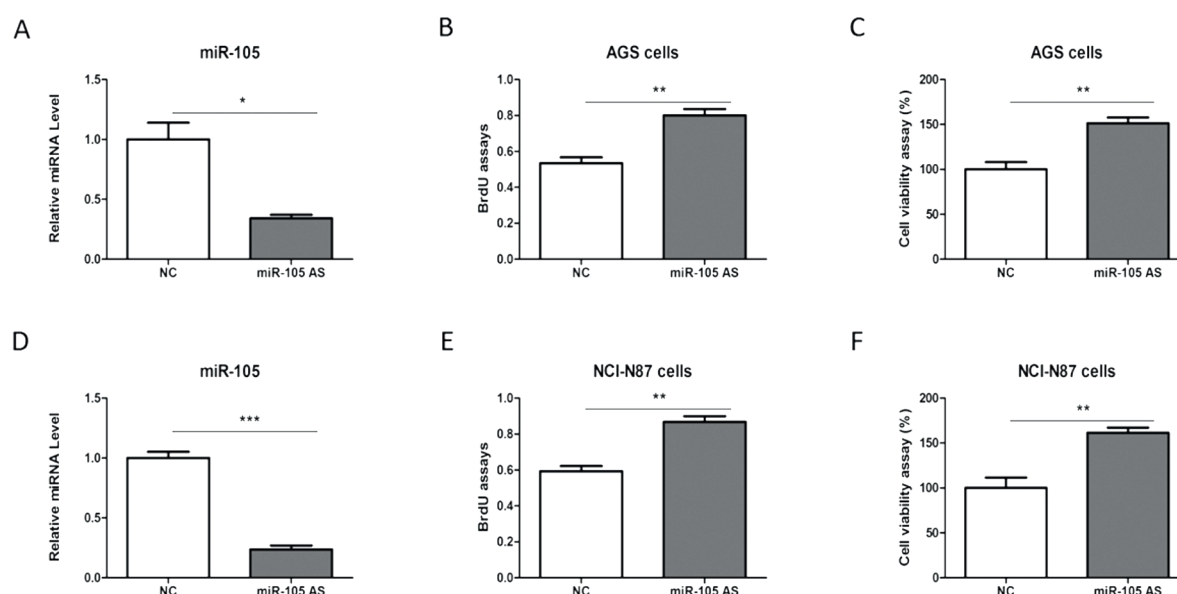


Figure 4. Knockdown of miR-105 promotes cell proliferation. **A**, Relative expression levels of miR-105 in AGS cells transfected with miR-105 antisense (miR-105 AS) or negative control (NC). **B-C**, Cell proliferation and viabilities in AGS cells transfected with miR-105 antisense or negative control (NC). **D**, Relative expression levels of miR-105 in NCI-N87 cells transfected with miR-105 antisense or negative control (NC). **E-F**, Cell proliferation and viabilities in NCI-N87 cells transfected with miR-105 antisense or negative control (NC).

two DNMTs, inhibited the expression of miR-105. Moreover, ectopic overexpression of miR-105 mimics inhibited, whereas its antisense pro-

moted cell proliferation and invasion in gastric cancer cells. Also, luciferase reporter assays and Western blot analysis found that miR-105 could

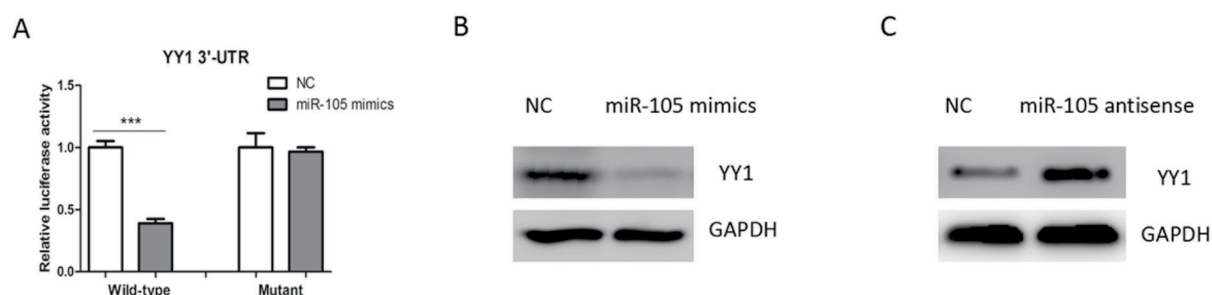


Figure 5. miR-105 inhibits YY1 expression. **A**, Relative luciferase reporter assays using wild-type or mutant 3'-UTR of YY1 in AGS cells transfected with miR-105 mimics or negative control (NC). **B-C**, Representative protein levels of YY1 in AGS cells transfected with miR-105 mimics, antisense or negative control (NC).

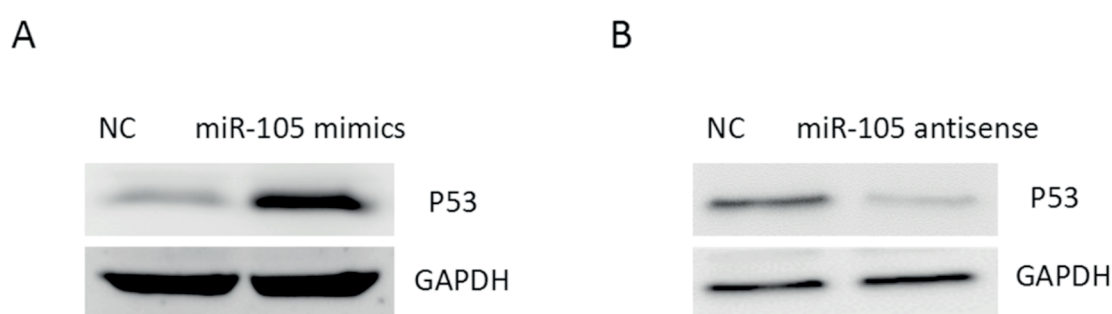


Figure 6. Regulation of p53 by miR-105. **A-B**, Representative protein levels of p53 in AGS cells transfected with miR-105 mimics, antisense or negative control (NC).

interact with 3'-UTR of YY1 gene, to inhibit its protein expression.

Previous researches have shown that miR-105 plays an important role in other types of human cancers. For instance, miR-105 is shown to inhibit prostate cancer growth by suppressing CDK6 levels¹⁴. Besides, miR-105 suppresses cell proliferation and inhibits PI3K/AKT signaling in human hepatocellular carcinoma¹⁵. However, some studies^{16,17} also demonstrated that miR-105, characteristically expressed and secreted by metastatic breast cancer cells, is a potent regulator of migration through destroying vascular endothelial barriers. Although the inconsistency remains unknown now, the expression and role of miR-105 in tumorigenesis might be cell- or tissue- specific.

It has been well-established that YY1 has a critical role in multiple biological processes, such as cell proliferation, differentiation, and cellular metabolism^{18,19}. In tumors, YY1 has been found to regulate the expression and/or function of many tumor suppressors or oncoproteins, including p53, c-Myc, and HIF-1^{12,13,20-22}.

Conclusions

We found that protein levels of p53 were regulated by miR-105, suggesting that the anti-proliferative roles of miR-105 might be dependent on the regulation of YY1-p53 axis. Notably, recent studies showed that YY1 could be targeted by several miRNAs in tumors^{23,24}, suggesting that dysregulated miRNAs and their potential target genes is complex, which needs further investigations.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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