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Down-regulation of MicroRNA-205 promotes gastric cancer cell proliferation

W.-Z. YIN, F. LI, L. ZHANG, X.-P. REN, N. ZHANG, J.-F. WEN

Department of Gastroenterology, the Second Affiliated Hospital, Harbin Medical University, Harbin, Heilongjiang, China

Abstract. – OBJECTIVE: Increasing evidence indicates that MicroRNAs, a class of small RNA molecules, play crucial roles in tumorigenesis, through affecting cell proliferation, apoptosis and metastasis. The present study aimed to investigate the effect of miR-205 on gastric cancer cell proliferation.

MATERIALS AND METHODS: The expression of miR-205 was examined in the gastric cancer tissues and cell lines. BrdU incorporation assay was used to measure the cell proliferation. Western blot was performed to determine the protein expression.

RESULTS: miR-205 is significantly down-regulated in gastric cancer tissues, compared with adjacent normal tissues. Besides, miR-205 expression is associated with clinical and pathological characteristics of patients. *In vitro* studies further found that inhibition of miR-205 significantly promoted gastric cancer cell proliferation via cell-cycle progression. Further analyses indicated that miR-205 was able to repress oncoprotein Yin Yang 1 expression, through targeting its 3'-untranlated region.

CONCLUSIONS: Our data suggest that down-regulation of miR-205 may represent an important mechanism for the development of gastric cancer.

Key Words:
miR-205, Yin Yang 1, Tumorigenesis, Gastric cancer.

Introduction

Gastric cancer (GC) has become one of the leading causes of cancer-related death worldwide, including China[1,2]. Although extensive efforts have been made, the mechanism of gastric cancer progression remains poorly understood.

Recently, MicroRNAs (miRNAs), a class of small RNA molecules, have been well studied to play critical roles in many processes of tumor progression, such as cell proliferation, metastasis, apoptosis, angiogenesis and epithelial to mesenchymal transition (EMT)[3-5]. For instance, previous studies have found that a number of miRNAs, including miR-101, miR-126, and miR-218, were down-regulated in gastric cancer tissues, whereas miR-223, miR-107 and miR-221/222 were up-regulated[6-11].

It has been reported that miR-205 is frequently downregulated in several cancers, including oral, prostate and breast cancer[12-14]. Indeed, overexpression of miR-205 reduced the growth, colony-formation capacity, cell migration and invasiveness of breast cancer cells, suggesting that miR-205 might be a tumor suppressor[14]. However, its role in the pathogenesis of gastric cancer is still unclear.

In the current work, we investigated the expression of miR-205 in gastric cancer tissues, and further silence its expression in two human gastric cancer cell lines, which may help to identify its role in the progress and prognosis of clinical gastric cancer.

Materials and Methods

Tissue Samples and Cell Culture

Twenty-five pairs of gastric tumors and adjacent nonmalignant tissues from gastrectomy were obtained from male patients at our department. Written informed consent was obtained from each patient. Our study was approved by the Hospital Institutional Review Board. AGS and NCI-N87 cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen).

RNA Extraction and Quantitative Real-Time PCR

Total RNAs were isolated by the mirVana miRNA Isolation Kit (Ambion, Austin, TX,

Corresponding Author: Ji-Feng Wen, MD; e-mail: wenjifeng1978@163.com

USA), and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Dalian, China). In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Otsu, Shiga, Japan) on Light Cycler 480 (Roche, Basel, Switzerland).

Cell Proliferation Assay

Cell counts were estimated by trypsinizing the cells and performing analysis with a Coulter counter (Beckman Coulter, Fullerton, CA, USA). For BrdU incorporation assays, a cell proliferation enzyme-linked immunosorbent assay (Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Cell-cycle Analysis

Cells were labeled for 15 min with PI and immediately analyzed by flow cytometry. Histograms represent the percentage of cells in each phase of the cell cycle (G0/G1, S and G2/M).

Western Blot

Cells or tissues were lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.0, 1% w/v SDS, 10% glycerol). After centrifugation at 4°C, proteins in the supernatants were quantified and separated by 10% SDS PAGE, transferred to NC membrane (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling, Danvers, MA, USA). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions. Anti-YY1 and C-myc antibodies were purchased from Abcam Company (Cambridge, MA, USA). Protein levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

Differences between groups were analyzed using Student's *t*-test analysis and expressed as the mean ± SEM from at least three separate experiments. Data were considered to be statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**) or $p < 0.01$ (***).

Results

miR-205 is Down-Regulated in Gastric Cancer Tissues

First, to examine the miRNA expression profile in gastric cancer, miRNA microarrays were performed in three pairs of primary gastric tumors and adjacent nonmalignant tissues. Among them, we found that miR-205 was the most down-regulated miRNAs (Data not shown). Next, to validate the microarray data, we performed quantitative real-time PCR analysis. As a result, we found that miR-205 expression were decreased in 84% (21/25, $p < 0.05$) of gastric tumors (Figure 1A).

Moreover, through analyzing the relationship between miR-205 expression and clinical characteristics of patient samples, we found that miR-205 expression level was significantly correlated with the progression of tumor invasion ($p < 0.01$, Figure 1B).

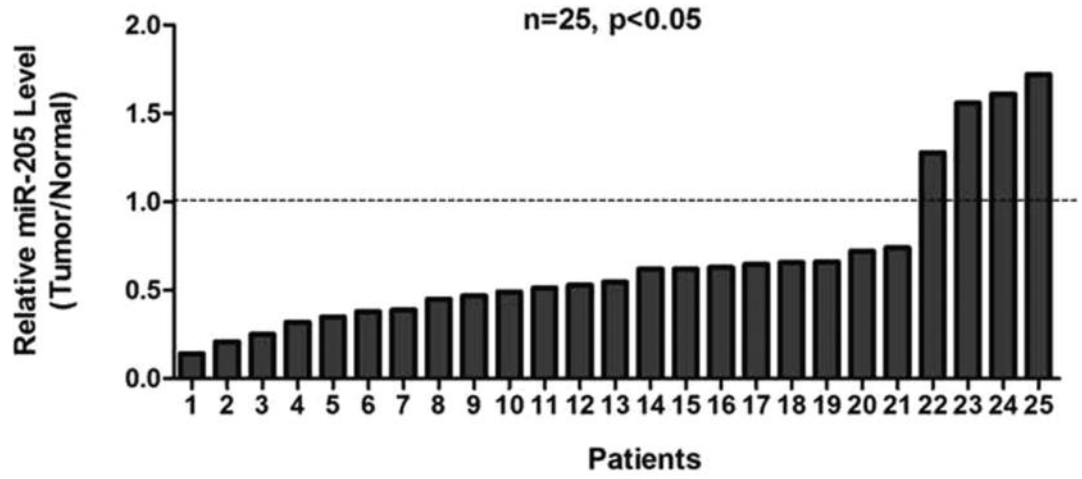
miR-205 Deficiency Promotes Gastric Cancer Cell Proliferation in vitro

To investigate the function of miR-205 in gastric cancer cells, loss-of-function experiments by introducing its antisense oligonucleotide into gastric cancer cells were employed (Figure 2A). As a result, cell growth was significantly increased in AGS cells where miR-205 was silenced (Figure 2B). Moreover, these cells had a higher rate of proliferation (Figure 2C). Consistently, cell-cycle analysis demonstrated that miR-205 deficiency led to a significant reduction in G0/G1 phase and increase in S phase (Figure 2D). Notably, similar results were also observed in NCI-N87 cells (Figure 3A-3D). Therefore, our results suggest that miR-205 inhibition could promote gastric cancer cell proliferation *in vitro*.

miR-205 Targets YY1 3′UTR and Down-Regulates its Expression

It has been known that miRNAs could regulate genes expression mainly through binding to the 3'-untranslated region (3'-UTR) of target mRNA[3-5]. Thus, we predicted the targets of miR-205 using software programs including TargetScan and miRBase, and found that Yin Yang 1 (YY1) was one of the targets identified by the two algorithms. Bioinformatics analysis revealed that there was one putative miR-205 binding site in the nucleotide sequence of YY1 3'-UTR (Figure 4A). To investigate whether YY1 is negatively regulated by miR-205, we performed Western

A



B

Characteristic variables	Down-regulated miR-205 expression (n=21)	p value
Age		
⩾60	12	
<60	9	0.24
Differentiation		
Well	6	
Moderate	8	
Low	7	0.57
Invasion depth		
T1	2	
T2	1	
T3	8	
T4	10	0.008
Lymph node metastasis		
Absent	12	
Present	9	0.19

Figure 1. Expression levels of miR-205 in gastric cancer tissues. ***A,*** miR-205 expression was determined by real-time PCR in human gastric cancer tissues and adjacent noncancerous tissues. ***B,*** Clinical and pathological characteristics of included patient samples and miR-205 expression.

blotting experiments, which revealed that YY1 protein levels were substantially elevated in the cells transfected with miR-205 antisense (Figure 4B-4C). Consistently, C-myc, a transcriptional target of YY1, was also up-regulated by miR-205 antisense (Figure 4B-4C).

To further verify whether YY1 is a direct target of miR-205, we constructed luciferase report vectors that contain the putative miR-205 binding sites within the YY1 3'-UTR. As shown in Figure 3F, overexpression of miR-205 mimics significantly repressed the luciferase activity, while miR-205 antisense enhanced the activity in AGS cells (Figure 4D). However, mutation of the miR-205 binding site from the YY1 3'-UTR abolished the effects of miR-205 mimics or antisense, sug-

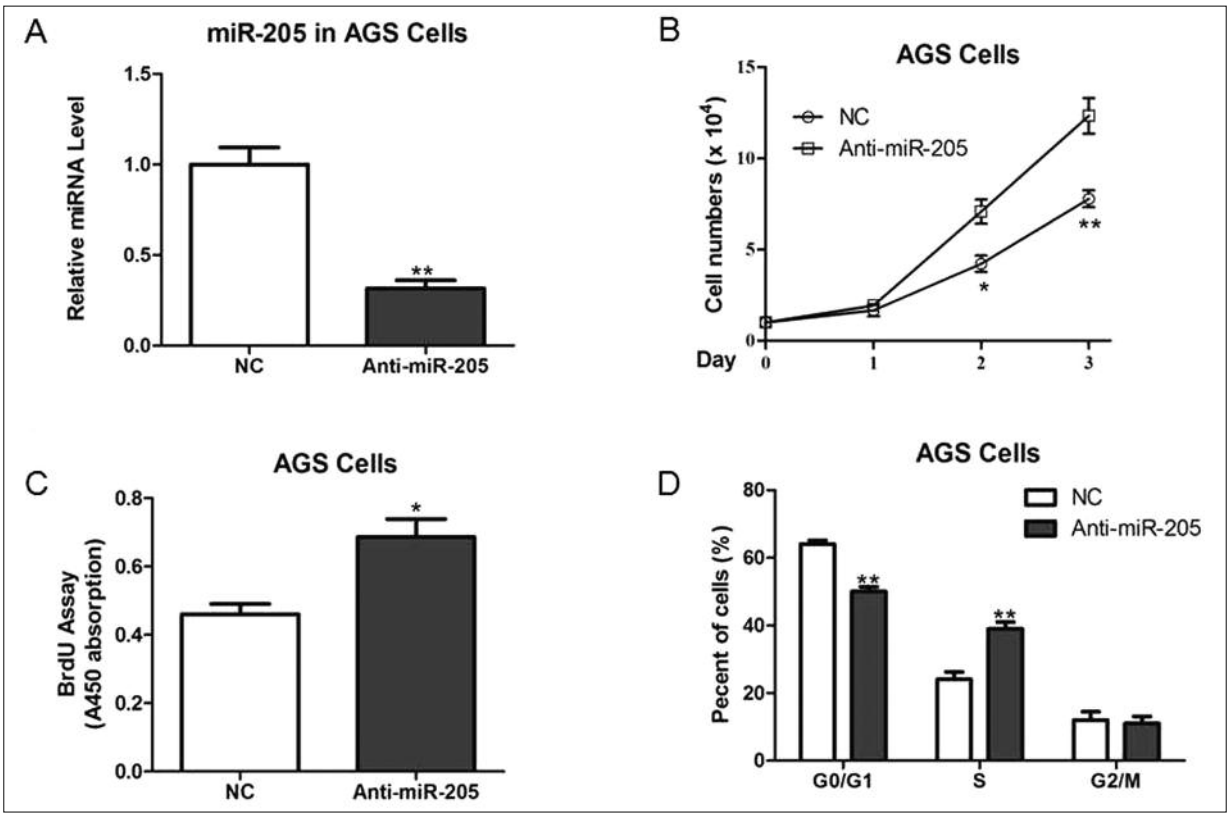


Figure 2. Knockdown of miR-205 enhances gastric cancer cell proliferation. ***A,*** Relative expression levels of miR-205 in AGS cells after transfection of miR-205 antisense (anti-miR205) or negative control (NC). ***B,*** The growth of AGS cells after transfection of miR-205 antisense (anti-miR205) or negative control (NC). ***C,*** The proliferative potential (BrdU) assays were determined in AGS cells transfected with miR-205 antisense or negative control (NC). ***D,*** The cell cycle phase of AGS cells transfected with miR-205 antisense or negative control (NC) were analyzed by flow cytometry.

gesting that miR-205 could directly repress YY1 expression by targeting its 3'-UTR (Figure 4D). Therefore, our results indicate that the oncoprotein YY1 represents a target gene of miR-205 in gastric cancer.

Discussion

Previous studies have reported that miR-205 could regulate cell proliferation and apoptosis through targeting several proteins, such as Bcl-2, PTEN and ERBB2[15,16]. In the present study, YY1 was validated as a novel target of miR-205 in gastric cancer. YY1 belongs to a transcription factor of the polycomb group protein family, which plays critical roles in the development, lipid metabolism and tumorigenesis[17,18]. In human cancers, aberrant expression of YY1 drives cell proliferation through up-regulation of C-myc and inhibition of tumor suppressor P53[18]. Several miRNAs appear to be involved in the regulation of YY1 signaling pathway. For instance, YY1 overexpression was found to be a consequence of miR-193a-5p down-regulation through direct miR-193a-5p-YY1 axis in human endometrioid endometrial adenocarcinoma[19]. Besides, miR-7 directly bound to YY1 3'-UTR to negatively regulate its protein abundance in colon cancer cell lines HCT116 and LOVO[20].

Here, we found that the down-regulation of miR-205 could increase YY1 expression in gastric cancer cells. However, until now, the mechanism underlying miR-205 down-regulation remains unknown. It was shown that miR-205 is directly transactivated by oncosuppressor p53 in breast cancer cells[21]. Besides, the hypermethylation of miR-205 promoter was found in hepatitis B virus-induced hepatocellular carcinoma[22]. Therefore, we will further investigate whether these factors may contribute to the down-regulation of miR-205 in gastric cancer in the future.

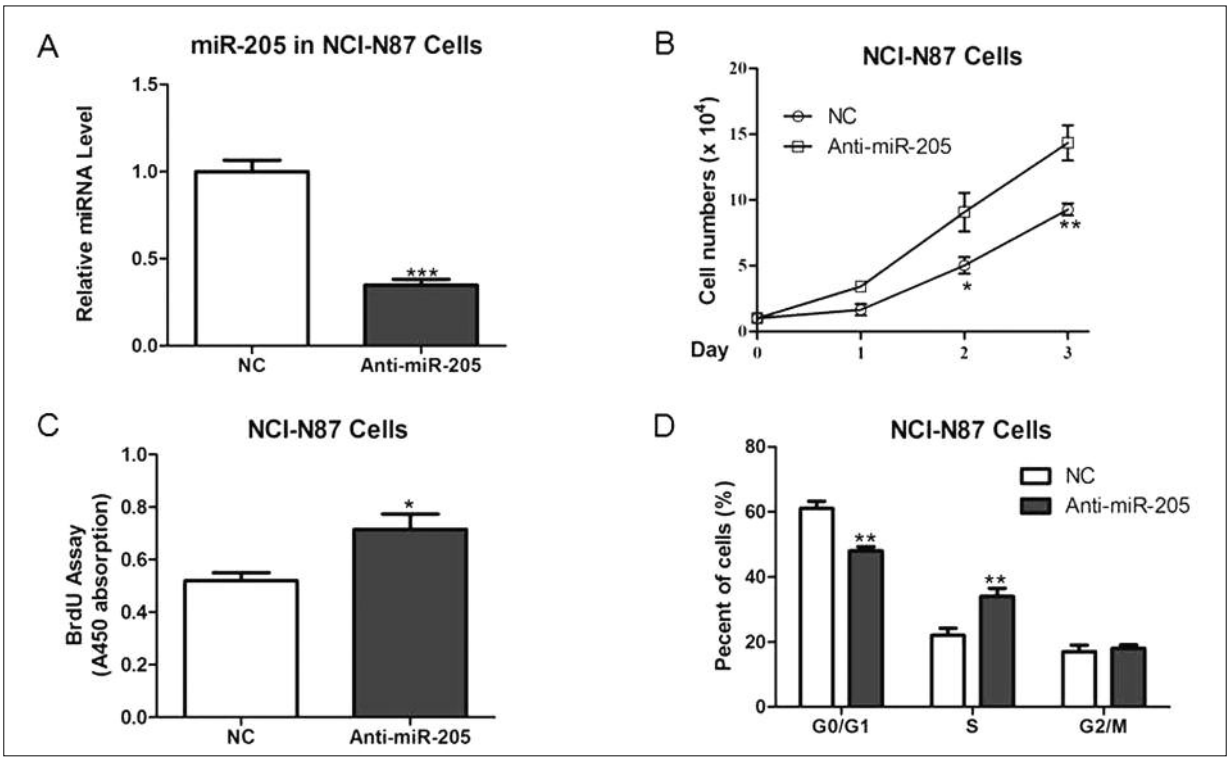


Figure 3. Knockdown of miR-205 enhances NCI-N87 cell proliferation. ***A,*** Relative expression levels of miR-205 in NCI-N87 cells after transfection of miR-205 antisense (anti-miR205) or negative control (NC). ***B,*** The growth of NCI-N87 cells after transfection of miR-205 antisense (anti-miR205) or negative control (NC). ***C,*** The proliferative potential (BrdU) assays were determined in NCI-N87 cells transfected with miR-205 antisense or negative control (NC). ***D,*** The cell cycle phase of NCI-N87 cells transfected with miR-205 antisense or negative control (NC) were analyzed by flow cytometry.

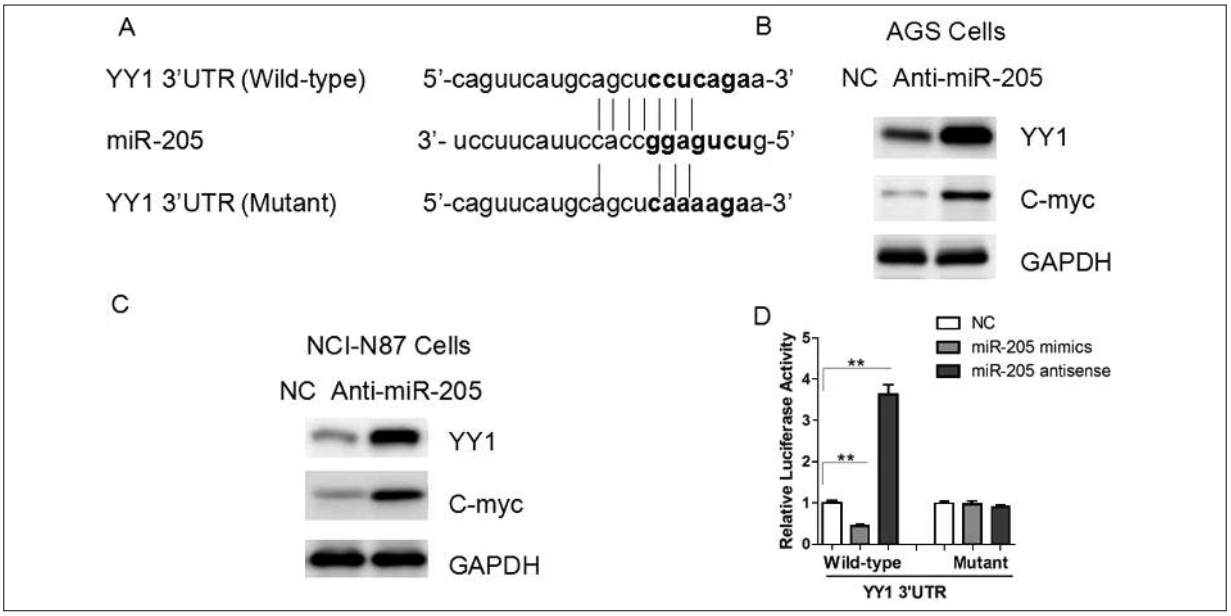


Figure 4. miR-205 targets YY1 3'UTR and down-regulates its expression. ***A,*** Prediction of miR-205 binding sites in the 3'UTRs of human YY1 gene by bioinformatic softwares. Potential binding site was highlighted in bold. ***B-C,*** Protein levels of YY1 and C-myc were determined by western blot in AGS and NCI-N87 cells transfected with miR-205 antisense or negative control (NC). ***D,*** Luciferase reporter assays in AGS cells. Cells were transfected with wild-type or mutant 3'-UTR-reporter constructs together with miR-205 mimics, miR-205 antisense or negative control (NC).

Conclusions

Our data highlights the notion that the down-regulation of miR-205 promotes gastric cancer cell growth *in vitro* by increasing YY1 expression. The functional interaction of miR-205 and YY1 signaling might help to explore the possibility of miR-205 in the diagnosis and treatment of gastric cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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