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miRNA-205 affects infiltration and metastasis of breast cancer

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ABSTRACT

Background: An increasing number of studies have shown that miRNAs are commonly deregulated in human malignancies, but little is known about the function of miRNA-205 (miR-205) in human breast cancer. The present study investigated the influence of miR-205 on breast cancer malignancy.
Methods: The expression level of miR-205 in the MCF7 breast cancer cell line was determined by quantitative (q)RT-PCR. We then analyzed the expression of miR-205 in breast cancer and paired non-tumor tissues. Finally, the roles of miR-205 in regulating tumor proliferation, apoptosis, migration, and target gene expression were studied by MTT assay, flow cytometry, qRT-PCR, Western blotting and luciferase assay.
Results: miR-205 was downregulated in breast cancer cells or tissues compared with normal breast cell lines or non-tumor tissues. Overexpression of miR-205 reduced the growth and colony-formation capacity of MCF7 cells by inducing apoptosis. Overexpression of miR-205 inhibited MCF7 cell migration and invasiveness. By bioinformation analysis, miR-205 was predicted to bind to the 3′ untranslated regions of human epidermal growth factor receptor (HER)3 mRNA, and upregulation of miR-205 reduced HER3 protein expression.
Conclusion: miR-205 is a tumor suppressor in human breast cancer by post-transcriptional inhibition of HER3 expression.

1. Introduction

Breast cancer is a common malignant tumor in women. In 2011, 230, 480 women were diagnosed with breast cancer, which accounted for 30% of female primary malignant tumors [1]. According to the statistics, the incidence of breast cancer for females in China remains 21.6 per 100,000 [2]. It is supposed that the high incidence of breast cancer could be attributed to both the genetic and non-genetic factors [3].

miRNAs are a class of small noncoding RNA molecules, which are considered to be key regulators in the process of cell development and division [3]. Under normal physiological conditions, miRNA is greatly involved in a series of cellular signaling pathways. Furthermore, miRNA interacts with the 3′-untranslated regions (UTRs) of their target genes, which silences the genes at the transcriptional and translational level [3]. miRNAs regulate the key signaling pathways in different cells, and changes in their expression greatly influence the protein expression patterns in cells, and cause a series of physiological and pathological transformations [4]. At present, in many diseases, the abnormal expression of miRNA has been widely reported [5]. Especially in cancer, the abnormal expression of miRNA is usually closely related to malignant proliferation and migration of cancer cells. As one of the most common cancers in women, abnormal expression of miRNA in breast cancer cell lines directly result in changes in the cell cycle and cell proliferation [6].

In breast cancer cells and tissues, we showed that expression of miR-205 was significantly downregulated. According to the selected pathological tissue samples, we found that downregulation of miR-205 was closely related to international gynecological tumor staging and lymph node transfer in patients with breast cancer and to lymph node metastasis. Our study provides experimental evidence suggesting that miR-205 is involved in the progression of breast cancer.

2. Materials and methods

2.1. Human samples and cell lines

A total of 60 primary human breast cancer and normal samples were collected at the First Affiliated Hospital of Medical College, Xi'an Jiaotong University. MCF7 and MCF10A cells were purchased from the American Type Tissue Culture Collection and cultured in MEM with 10% fetal bovine serum.

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2.2. Transient transfection

Shortly before transfection, we seeded 0.4×10^5–1.6×10^5 cells/well in a 24-well plate in 0.5 mL MEM containing serum and antibiotics. For the short time until transfection, we incubated the cells under normal growth conditions (typically 37 °C and 5% CO_2). Then, miR-205 mimics, miR-205 inhibitor, or miR-negative controls (Genepharma) were pre-incubated with HiperFect transfection reagent (Qiagen) with a final concentration of miRNA analogs at 100 nmol/L. The sequence of miR-205 was as follows: 5′-UCCUUCAUUCCACCGGAGUCUG-3′.

2.3. RNA extraction

Total RNA was extracted from cell lines and human samples (5 mg) with Trizol Reagent (Invitrogen) according to the manufacturer's instructions.

2.4. Real-time PCR

To detect and quantify mature miR-205, TaqMan MicroRNA Reverse Transcription kit and TaqMan MicroRNA assay were used in accordance with manufacturer's instructions (Applied Biosystems). Normalization was performed with U6 RNA. For the quantitation of miRNA, 10 ng total RNA was reverse transcribed using Taq-Man MicroRNA Reverse Transcription Kit (Applied Biosystems) with specific primers for miR-205 and RNU48, and subsequently the PCR amplifications were performed in reaction volumes of 20 μL containing 10 μg TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 1 μL 20× TaqMan MicroRNA Assay Mix (Applied Biosystems) and 1.33 μL template cDNA in the same system used for mRNA quantitation. The thermal cycling conditions were, a hot start step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Relative miRNA expression of miR-205 was normalized against the endogenous control, U6 RNA, using the comparative ΔΔCt method. Bio-Rad CFX Manager Software was used for quantitation analysis for both mRNA and miRNA.

2.5. Protein extraction, Western blotting and antibodies

The cell lysates were separated by 10% SDS–PAGE and transferred electrophoretically to a PVDF membrane. After blocked with 5% dry milk in PBS, the membranes were probed with the human epidermal growth factor receptor (HER)3 antibody (SantaCruz Biotechnology). The appropriate HRP-conjugated secondary antibodies were subsequently applied and immunodetection achieved using the ECL plus detection system (GE Healthcare), followed by fluorescence detection using ChemiDoc XRS-J imaging.

2.6. Luciferase target assay

For the luciferase assay, the 3′-UTR of HER3, including the binding site for miR-205, was amplified from MCF7 cells by using the following primers: ERBB3-L: 5′-AATTTCTAGAGTAACTCCTGCTCCCT GTGG-3′ and ERBB3-R: 5′-AATTTCTAGATGAATTTGCCCTCGGATA AG-3′. The PCR product was then digested with *Xba*I and cloned into the reporter plasmid pGL3 (Promega) downstream of the luciferase reporter gene.

The modified firefly luciferase vector (500 ng/μL) was transfected into HEK293 cells (2×10^5 cells/mL), as previously described [10]. Firefly and Renilla luciferase activities were measured 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Firefly activity was normalized to Renilla activity to control the transfection efficiency.

2.7. Dimethyl thiazolyl diphenyl tetrazolium (MTT) assay

To evaluate the effect of miR-205 on cell proliferation, cells were seeded at 5000/well in 100 μL medium in 96-well plates and transfected with miR-205 mimics (50 nM) and negative control-miRNA mimics (50 nM), as described above. Every 24 h post-transfection, 20 μL MTT reagent (Solarbio) was added to the wells and they were incubated for 4 h. After removing the medium, 200 μL DMSO was added to dissolve the formazan and the absorbance was measured at 490 nm. Wells containing only MCF7 cells served as blanks.

2.8. Scratch assay

Cells were grown as a confluent monolayer in six-well plates. To initiate migration, the cell layer was scratched using a pipette tip. Next, the cells were transfected with antago-miR-205 or negative control. Time-lapse images of cell morphology were captured at 24 h and 48 h. The migration abilities were quantified by measuring the area of the scratched regions using the ImagePro Plus 4.5 software. The experiment was performed three times.

2.9. Apoptosis assay

Flow cytometry was performed to detect apoptosis using an annexin V–FITC Apoptosis Detection Kit (Bio-vision). MCF7 cells were transfected with miR-205 mimics (50 nM) and a negative control (50 nM). The samples were analyzed by flow cytometry (FACS, BD).

2.10. Statistical analysis

Data were presented as mean ± SE from three independent experiments. Statistical analysis was carried out with Student's *t* test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. MiR-205 is downregulated in breast cells and tissue samples

To detect the expression of miR-205, a real-time quantitative (q)RT-PCR was performed in MCF7 human breast cancer cells, and U6 small nuclear RNA was used as an internal control. We found that miR-205 was downregulated by >70% in MCF7 breast

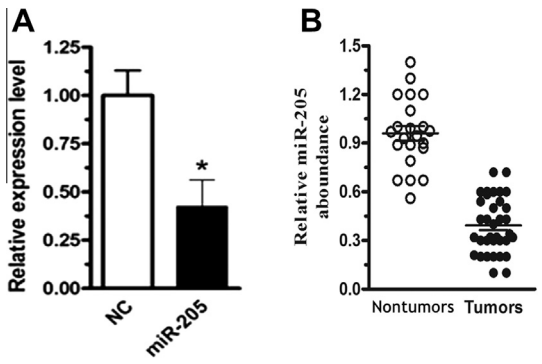

Fig. 1. Expression of miR-205 in breast cancer cells or tissues. (A) qRT-PCR analysis of miR-295 expression in breast cancer cell line (MCF7) and normal breast epithelial cells (MCF10A). (B) qRT-PCR analysis of miR-205 expression in 60 cases of breast cancer and paired non-tumor tissues. U6 was used as an endogenous control. Expression levels of miRNAs were determined in individual sample by RT-qPCR. Fold changes compared with the control group were calculated based on the ΔCt method. Data represented the means ± SEM and statistical significance were tested by Student's *t*-test, $n = 3$ independent experiments. $^*p < 0.05$ versus control.

cancer cells when compared with normal breast cells (MCF10A) (Fig. 1A; $p < 0.01$). Expression of miR-205 was explored in 60 pairs of breast cancer tissues and corresponding non-tumor tissues. The mean miR-205 expression level was 0.54 ± 0.22 for breast cancer tissues and 1.09 ± 0.17 for paired non-tumor tissues. Thus, miR-205 was downregulated in breast cancer tissues compared with non-tumor tissues (Fig. 1B; $p < 0.01$).

3.2. Overexpression of miR-205 inhibits growth, enhances apoptosis, and decreases invasion of breast cancer cells

To study the function of miR-205 in breast cancer, miR-205 mimics or negative controls were transiently transfected into MCF 10A cells. At 48 h after transfection, compared with negative-control-transfected cells, miR-205 expression level was increased by 26–32 times ($p < 0.01$; Fig. 2A) in MCF10A cells transfected with miR-205 mimics. MTT and colony formation assays were conducted to analyze the cell proliferation at 0, 24, 48 and 72 h after transfection. According to Fig. 2B, cell growth rate was significantly lowered to 47% and 56% compared with negative controls at 48 and 72 h, respectively ($p < 0.05$). Furthermore, cell migration assay was also performed by scratching the cell layer prior to transfection with antoga-miR-205 or negative control. Obviously, downregulation of miR-205 significantly enhances cell migration (Fig. 2C). In addition, an Annexin V and propidium iodide kit was used to determine the rate of apoptosis through flow cytometry. According to the statistics, 124% more apoptosis was detected in MCF7 cells transfected with miR-205 mimics.

3.3. MiR-205 directly targets HER3 in human breast cancer cells

Based on miR target analysis (TargetScan and miRBase), miR-205 was predicted to target HER3, which was usually highly expressed in cancer tissues. To determine the effect of miR-205

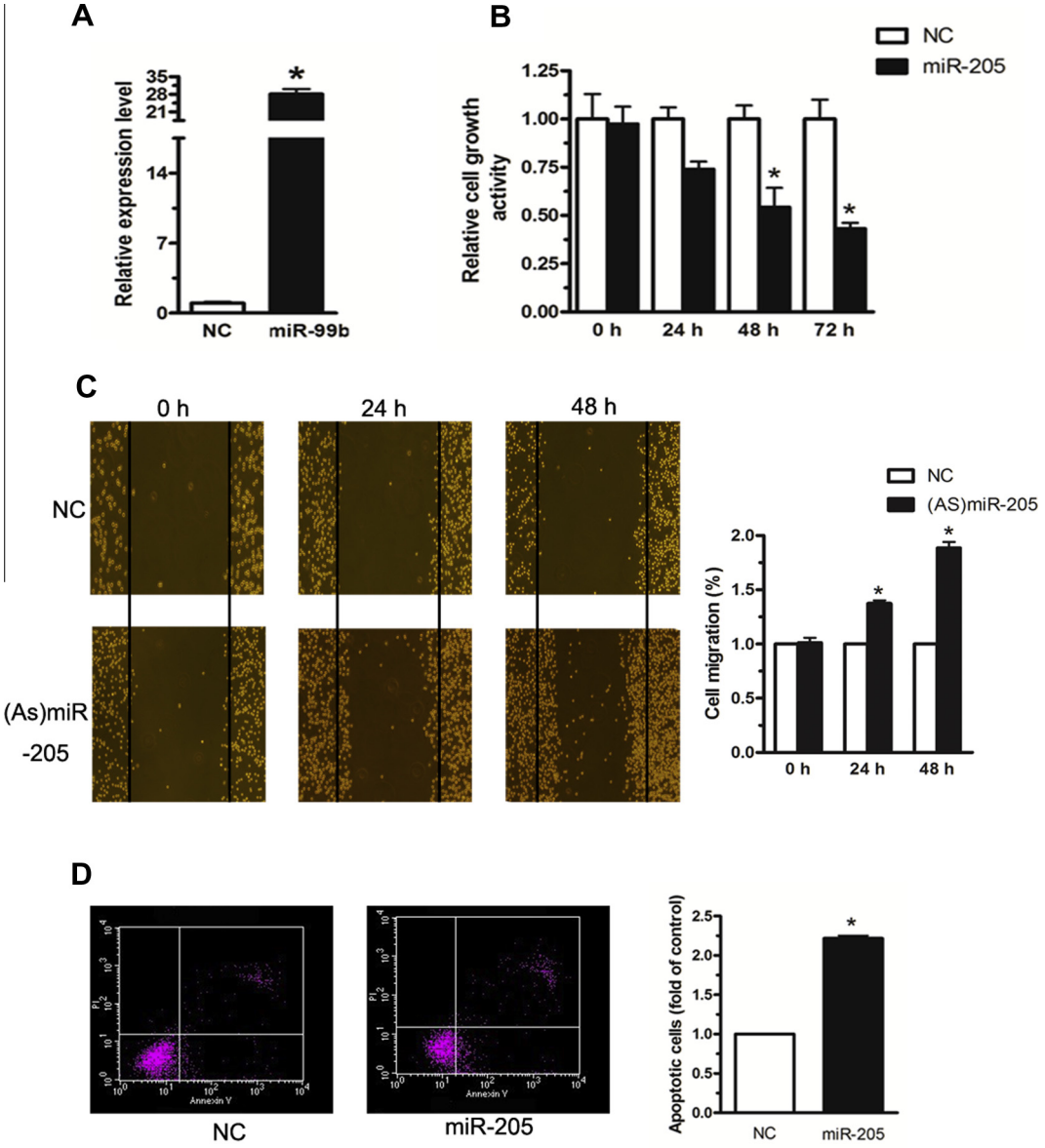

Fig. 2. Effects of miR-205 expression on growth, colony formation, and apoptosis in breast cancer cell lines. (A) Transfection efficiency of miR-205. (B) MTT analysis of relative growth activity of MCF7 cells transfected with miR-205 mimics at different time points (0, 24, 48 or 72 h). (C) Cell migration assay was performed by scratching the cell layer prior to transfection with antago-miR-205 or negative control. Time-based images were obtained from 0 to 48 h. The migration abilities were quantified by measuring the area of the scratched regions using the ImagePro Plus 4.5 software. (D) Flow cytometry analysis of MCF7 cell transfected with miR-205 mimics. Data represented the means ± SEM and statistical significance were tested by Student's *t*-test, $n = 3$ independent experiments. $^{*}p < 0.05$ versus control.

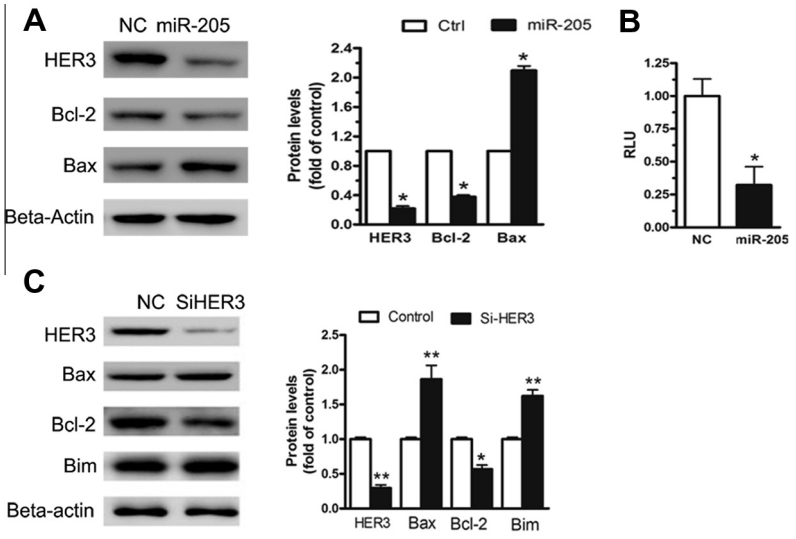

Fig. 3. miR-205 directly targets HER3 in breast cancer cells. (A) Western blot analysis of HER3 protein expression in negative control or miR-205 mimics-transfected MCF7 cells. (B) HEK293 cells were cotransfected with miR-205 mimics or negative control and luciferase vector pGL3 containing HER3/3′-UTR sequence. After 24 h, the luciferase activity was measured. Values are presented as relative luciferase activity after normalization to Renilla luciferase activity. (C) Western blotting illustrating that si-HER3 decreased Bcl-2 protein expression, and stimulated the level of Bax and Bim.

on HER3 protein expression, Western blot analysis was performed at 48 h after transfection of miR-205 mimics into MCF10A cells. Fig. 3A shows that the expression level of HER3 protein was reduced in MCF 10A cells transfected with miR-205 mimics. At the same time, the antiapoptotic protein, Bcl-2, was reduced and the proapoptotic protein, Bax, was enhanced (Fig. 3A). To confirm that miR-205 targets HER3, a firefly luciferase reporter assay was conducted. The 3′-UTR of HER3 was cloned into firefly luciferase reporter vector. The HER3 luciferase constructs (HER3/3′-UTR or HER3/3′-UTR-del) were then cotransfected with miR-205 mimics or negative control into HEK293 cells. miR-205 overexpression reduced HER3/3′-UTR luciferase activity (Fig. 3B). According to the data, miR-205 targets HER3 in human breast cancer cells. In order to investigate the effect of HER3 on apoptosis, we knocked down the expression level of HER3 in MCF10A cells. As supposed, in siRNA-HER3-transfected cells, total Bcl-2 level was lower than the corresponding control cells, whereas Bax and Bim levels were raised.

4. Discussion

Apoptosis is triggered by either intrinsic or extrinsic factors. The extrinsic pathway is related to the stimulation of the transmembrane death receptors, including Fas, tumor necrosis factor receptor 1, and Apo2/Apo3, while the intrinsic pathway is regulated by signaling factors released from the mitochondria. Blocking or delaying cell death is a major mechanism by which pathogens promote intracellular survival and replication [7]. In recent years, dysregulation of miRNA has been increasingly attracting the attention of researchers and clinicians [8]. Many researchers have suggested that miRNA is closely related to human malignant cancer, such as breast cancer [9]. Changes in miRNA have been reported to exert a direct effect on malignant transformation, metastasis, and
chemo- or radioresistance [10]. Our results suggest that miR-205 is downregulated in human breast cancer tissues and MCF7 cells. We also showed that overexpression of miR-205 inhibited breast cell growth and invasion by regulating HER3 expression.

We detected the expression of miR-205 in breast cancer cell lines and tissue samples. Our data suggested that the expression level of miR-205 was significantly lower in breast cancer cells and tissues than in normal breast and corresponding non-tumor breast tissues. We conclude that the decreased expression of miR-205 might play a pivotal role in the progression and development of breast cancer.

To repress translation, miRNA could bind imperfectly to the 3′-UTR of target mRNAs. Thus, miRNAs can suppress protein expression at the translation level or post-transcription level [11]. Based on bioinformation analysis, miR-205 is predicted to target HER3. HER3 is a transmembrane receptor that is activated by human epithelial growth factor [12]. The binding of ligands to the receptor leads to receptor dimerization, activation of protein tyrosine kinase, intermolecular receptor autophosphorylation, and phosphorylation of cellular substrates that consequently enhances cell proliferation [13].

A high expression level of HER3 has been reported in a series of human cancers, including breast cancer. Researchers have suggested that HER3 significantly enhances the invasiveness and proliferation of breast cancer cells [12]. It is suggested that downregulation of HER3 enhances the expression level of Bax and reduces the level of bcl-2, which then cause apoptosis [12]. Apoptosis is induced when HER3–PI3K–AKT signaling is enhanced [14]. Our results suggest that knockdown of HER3 decreases the level of Bcl-2 and increases expression of Bax. Based on sequence analysis, the 3′-UTR of HER3 contains a putative binding site for miR-205. Luciferase reporter assay suggested that miR-205 decreased HER3/3′-UTR luciferase activity. Overexpression of miR-205 reduced HER3 protein levels. These data suggest that miR-205 directly targets HER3 in human breast cancer. It may partially contribute to the overexpression of HER3, which then causes the malignant proliferation and growth of breast cancer cells.

In conclusion, the present study showed that the expression level of miR-205 is dramatically downregulated in breast cancer cells and tissues. Through targeting HER3, overexpression of miR-205 could inhibit growth, enhance apoptosis, and decrease the migration capacity of breast cancer cells. It was demonstrated that miR-205 as a new oncosuppressor gene in breast cancer, which is able to interfere with the proliferative pathway mediated by the HER receptor family.

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