Original Article
Up-regulation of miR-506 inhibits cell growth and disrupt the cell cycle by targeting YAP in breast cancer cells

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Abstract: MicroRNAs (miRNAs) are a small class of non-coding RNAs that are extensively deregulated in various cancers. They can act as either oncogenes or tumor suppressor genes in human cancer. The purpose of this study was to investigate the crucial role of miR-506 in breast cancer and to validate whether miR-506 could regulate proliferation of breast cancer cells by targeting YAP (Yes-associated protein) gene. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to quantify the expression levels of miR-506 in breast cancer and adjacent non-cancerous breast tissues. To characterize the miR-506 function, MTT assays, colony formation assays, cell migration assays, cell invasion assays and cell cycle assays were used. Finally, luciferase reporter assays were performed to validate the regulation of a putative target of miR-506, in corroboration with western blot assays. We found that expression of miR-506 was commonly down-regulated in breast cancer cells and breast cancer specimens when compared with that in non-malignant breast epithelial cells and adjacent normal tissues. Up-regulation of miR-506 inhibited cellular proliferation, migration and invasion as well as disrupt the cell cycle of breast cancer cells. Luciferase assays revealed that miR-506 directly bound to the 3'-untranslated region (3'-UTR) of YAP. Western blot analysis verified that miR-506 regulated the expression of YAP at the protein levels. These findings suggest that miR-506 exerts as a tumor suppressor in breast cancer and up-regulation of miR-506 expression inhibits cellular growth, cell migration and invasion as well as disrupt the cell cycle by targeting YAP. Our study demonstrates that the miR-506/YAP axis may help us better understand the molecular mechanisms of breast cancer progression.

Keywords: MiR-506, breast cancer, YAP

Introduction
microRNAs (miRNAs) are small, non-coding RNAs of approximately 19-25 nucleotides which are capable of regulating gene expression at the post-transcriptional level. Mechanistically, miRNAs play its role by binding to the 3'-untranslated regions (3'-UTRs) of certain mRNAs, causing translation to be blocked and/or mRNA degradation [1, 2]. In recent decades, miRNAs are found to be involved in many important physiological and pathological processes, such as cell proliferation, development, differentiation, virus infection and tumorigenesis, and are widely dysregulated in various cancers [3, 4]. Accumulating evidence suggests that miRNAs may function as either tumor-suppressor genes or oncogenes [5]. Previous studies showed that miRNA-506 (miR-506) was down-regulated in a series of tumors like breast cancer [6], hepatocellular cancer [7] and cervical cancer [8]. However, to date, the concrete role of miR-506 in breast cancer tumorigenesis is incompletely understood.

Breast cancer is the second-leading cause of cancer-related death in women. Although great progress has been made in the earlier diagnosis and systemic therapy in recent years, recurrence and distant metastasis remain to be major obstacles in the successful treatment of breast cancer, and the mechanism of breast occurrence is still not completely understood [9-11]. The Hippo pathway is demonstrated to be associated with mammary gland development and breast cancer [12]. It is acknowl-
edged that deregulation of this pathway leads to massive overgrowth of tissue and induces cancer in mammals [13]. As the most critical effector of the Hippo pathway, YAP (Yes-associated protein) plays a considerable role by co-activating key transcriptional factors [14, 15]. Accumulated evidence suggests that YAP is an oncprotein that promotes breast cancer tumorigenesis and progress. Consistently, YAP over-expression has been demonstrated to promote breast cancer cell growth in vitro and in vivo [16]. What’s more, YAP has also been shown to promote breast cell migration, invasion, epithelial-to-mesenchymal (EMT) transition, and metastasis [17]. However, further understanding of the post-transcriptional control of the YAP gene in breast cancer remains elusive.

In this study, we aim to investigate the function of miR-506 in breast cancer. Interestingly, our experiments indicate that miR-506 is able to suppress the proliferation of human breast cancer cells in vitro by direct targeting YAP mRNA. Our findings provide new insights into the mechanism by which miR-506 modulates cell proliferation in breast cancer.

Materials and methods

Specimens

In this study, 12 pairs of breast cancer and adjacent normal specimens were collected from the Department of Breast and Thyroid Surgery of Shanghai Tenth People’s Hospital, Shanghai, China. The samples were immediately snap-frozen in liquid nitrogen. All the samples were confirmed as invasive ductal breast cancer, and no patients had received any chemotherapy or radiotherapy prior to surgery.

Cell culture

The MDA-MB-231 breast cancer cell line, MCF-7 breast cancer cell line and MCF-10A cell line were purchased from Chinese of Sciences in Shanghai. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Enpromise, China). Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO2.

RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

According to the manufacturer’s protocol, total RNA was extracted from the cells or tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). For detection of miR-506 expression, primer design and qRT-PCR were carried out according to the manufacturer’s instructions. The primers used were as followed: miR-506 forward, 5'-TAAGGCACCCTTCTGAGTAGA-3'; reverse, 5'-GCCAGCAGAATTAATACGAC-3'; U6 forward, 5'-AGAGCCTGTGGTGTCCC-3'; reverse, 5'-CATCTTCAAGCAGCTCCT-3'. cDNA was generated by reverse transcription using the PrimeScript™ RT-PCR kit in accordance with the manufacturer’s instructions (Takara, Tokyo, Japan). Real-time PCR was performed on a 7900HT Fast RT-PCR instrument (Applied Biosystems, Singapore). The amplification procedure was as follows: 5 min at 95°C, followed by 40 cycles at 95°C for 30 sec and 65°C for 45 sec. The relative expression was evaluated following the relative quantification equation, 2^(-ΔΔCt). Each sample was tested in triplicate.

Transfection assay

Cells (1 x 10⁶) were added into each well of a 6-well plate and cultured with DMEM medium without serum and antibiotics. As the confluency of MDA-MB-231 breast cancer cells reached 80-90%, miR-506 mimics and lipofect at the ratio of 1 μg: 3 μl were diluted to 250 μl by DMEM medium, respectively, and incubated for 5 min at room temperature. MiR-506 mimics and the lipofect dilution were gently combined and incubated for 20 min. Subsequently, 500 μl of the complexes were added to each well. After 4-5 h of incubation, DMEM medium was replaced by DMEM with 10% FBS, and all the cells were incubated at 37°C in a CO₂ incubator for 48 h prior to further testing.

Western blot analysis

Cell protein was extracted by using RIPA lysis buffer. The supernatant was quantified by bicinchoninic acid assay (Pierce, USA). Subsequently, 30 μg of protein samples was denatured with 5X sodium dodecyl sulfate (SDS) loading buffer at 95°C for 5 min. Next, whole protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferr-
ed onto 0.45-μm nitrocellulose membranes (Beyotime). After 1 h of blocking in 5% fat-free milk, the membranes were incubated with the YAP antibody (1:1,000) and the β-actin antibody (1:1,000) (both from Cell Signaling Technology, USA) overnight at 4°C. Protein blots were washed and then incubated for 1 h with specific secondary antibodies. After washing by PBST for 3 times, immunoreactive protein bands were detected using the Odyssey scanning system (LI-COR, Lincoln, NE, USA).

**Cell viability assay**

Cells were plated in 96-well plates (BD Biosciences, USA) at 2 × 10^3 cells per well 24 h post-transfection. When the cells reached 30-40% confluence, they were transfected with either 50 or 100 nM miR-506 mimics or NC mimics. We assessed cell proliferation at 24, 48, 72 and 96 h post-transfection using the MTT assay. Briefly, 20 μl (5 mg/ml) MTT (Sigma, USA) solution was added to each well. After a 4-h incubation at 37°C, the supernatant was removed and 150 μl DMSO was added. After 10 min of agitation (100 rpm), optical density at 490 nm was determined on a microplate spectrophotometer. Each sample was tested with six replicates. All experiments were performed in biological triplicate.

**Colony formation assay**

Three hundred cells of each group (miR-506 and NC) were plated in a 6-well plate in complete medium. After 7-10 days, or when the colonies were visible by viewing with the eye, the culture was terminated. Then the plates were washed twice in phosphate buffered saline (PBS) after removing the complete medium. Then the colonies were fixed in 95% ethanol for 10 min, dried and stained with 0.1% crystal violet solution for 10 min. At last, each plate was washed three times with water, and the number of colonies was counted only if the well contained >50 cells. The experiment was performed three times.

**Wound healing assay**

In the in vitro wound healing assay, transfected MDA-MB-231 cells were grown in 6-well plates until the cell confluence reached about 90%. Then the plates were washed twice in PBS after making a scratch in each well using a sterile pipette tip, and cells were then maintained at 37°C in an atmosphere with 5% CO₂. Wound healing was observed under a light microscope and images were captured at the same view at 0, 12, 24 and 48 h after scratching to observe the process of wound healing. The experiments were repeated twice and representative photographs are shown.

**Invasion assay**

A transwell invasion assay was performed in miR-506 mimics group and control group with CHENICON Cell Invasion Assay Kit (Chemicon, USA). Transwell chambers were pre-coated with Matrigel. We added cells to the Transwell system 48 h after the transfection. The lower chambers were filled with 500 μl of DMEM containing 15% FBS. Then the Transwell system was placed in the 24-well plate followed by the incubation for 48 h at 37°C in an atmosphere with 5% CO₂. The membrane was stained with 0.1% crystal violet and observed under a microscope after removing the Matrigel and cells in the upper chambers. Five fields were randomly selected from each membrane, and the number of cells penetrating the membrane was counted at a magnification of × 200. The invasion ability was described as the number of invading cells. The invasion of each group was assayed in triplicates and the experiment was repeated three times.

**Cell cycle assay**

Thirty-six hours after transfection with the miR-506 mimics, NC, or miR-506 inhibitor, cells were trypsinized and then centrifuged at 1,000 rpm for 5 min, followed by two washes in cold PBS. Then, 3.0 ml ice-cold ethanol was added in a dropwise fashion and cells were allowed to fix for more than 30 min. A total of 250 μl 0.05 g/l propidium iodide (PI) staining solution was added into each sample and incubated for 30 min at RT. Finally, cells analyzed on a flow cytometer (FACSCanto™ II, BD Biosciences).

**Statistical analysis**

Data from at least three separate experiments are presented as the means ± standard error of the mean (SEM). Student’s t test was used for comparisons. P values <0.05 were considered significantly different between groups.
Results

**MiR-506 is down-regulated in both human breast cancer specimens and breast cancer cell lines**

Deregulation of miRNA expression has been demonstrated to be related with tumor development and progression. It has been reported that miR-506 shows a significant difference with distant-relapse-free survival (DRFS) in almost all breast cancer patients [6]. However, the expression of miR-506 in breast cancer is still unclear. First, we analyzed the expression of miR-506 in 12 paired clinical breast cancer specimens and adjacent non-tumor breast tissues using qRT-PCR with normalization against an endogenous control (U6 RNA). All 12 breast cancer samples showed significant down-regulation of miR-506 compared with adjacent non-tumor tissues (Figure 1), indicating that low-level expression of miR-506 is a frequent event in breast cancer tissues. We also detect the expression of miR-506 in breast cancer cell lines MCF-7 and MDA-MB-231 compared to MCF-10A. The expression of miR-506 was analyzed by qRT-PCR. The graph represents the $2^{-\Delta\Delta C_T}$ values ± SD; *P<0.05.

**Forced expression of miR-506 suppresses the proliferation of breast cancer cells**

To investigate the impact of miR-506 on the proliferation and migration of breast cancer cells, miR-506 mimics and miR-506 inhibitor were transfected into MDA-MB-231 cells at the final concentrations of 100 nmol/L. We assessed cell proliferation at 24, 48, 72 and 96 h post-transfection using MTT assays. Briefly, inhibition rate was calculated as following: inhibition rate (%) = (OD value of the control group - OD value of experimental group)/OD value of control group × 100%. Compared with the control group, miR-506 mimics group was inhibited in a dose and time dependent manner. Cell proliferation was strongest inhibited...
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Figure 4. Representative images of crystal violet stained colonies in MDA-MB-231 cells transfected with miR-506 mimics or inhibitor or miR-NC.

Figure 5. MiR-506 inhibited scratch healing of MDA-MB-231 cells. Cell scratch assay was used to detect the migration of MDA-MB-231 cells. Scratch healed in NC group after 48 h, while scratch in miR-506 transfected group did not.

when cells were treated with 100 nmol/L miR-506 mimics for 96 h (Figure 3). Colony formation assays also showed much less colony formation in the group transfected with 100 nmol/L miR-506 mimics compared with the NC group and miR-506 inhibitor group (Figure 4). These results revealed that forced overexpression of miR-506 suppressed the colony formation ability of MDA-MB-231 cells, indicating that overexpression of miR-506 suppressed cellular proliferation.

Overexpression of miR-506 inhibits cell migration and invasion of breast cancer cells

To research how overexpression of miR-506 affects cellular migration and invasion, we performed wound healing assays and transwell assays with MDA-MB-231 cells transfected with miR-506 mimics (100 nmol/L), NC mimics. The wound healing assay results showed that the migration ability of the miR-506 mimic group was lower than the NC group. As shown in Figure 5, 36 h after drawing the “scratch” line on the monolayer cells, the cell-free area of the miR-506 mimic group was apparently wider than the NC group, and when the NC group filled in the gap at 48 h, the miR-506 group still showed a clear gap in the scratched region. The results indicate that overexpression of miR-506 in MDA-MB-231 cells inhibited cellular migration. The transwell invasion assay revealed that the number of MDA-MB-231 cells penetrating the membrane significantly
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decreased at 48 h after miR-506 transfection as compared to the control group and miR-506 inhibitor group (Figure 6). The number of cells penetrating the membrane was markedly lower in the transfected cells as compared to untransfected cells (P<0.05). Together these results showed that overexpression of miR-506 can suppress cellular migration and invasion ability.

MiR-506 disrupts the cell cycle of breast cancer cells

Thirty-six hours after the transfection of miR-506 mimics (100 nmol/l) and miR-506 inhibitor (100 nmol/l), flow cytometry analysis indicated that the percentage of G0/G1 phase cells (71.33%) dramatically increased in the miR-506 mimics group, when compared with that of the NC group (54.33%) and miR-506 inhibitor group (41.72%) (P<0.05), while the proportion of S-phase cells decreased in the miR-506 mimics group (17.52%) compared with that of the NC group (29.35%) and miR-506 inhibitor group (39.90%) (P<0.05). The percentage of G2/M phase cells also decreased in the miR-506 mimics group (11.15%) compared with that of the NC group (16.33%) and miR-506 inhibitor group (18.38%). These findings revealed that miR-506 can initiate G0/G1 phase arrest, upregulation of miR-506 expression could lead to the reduction of S-phase and G2/M phase cells (Figure 7).

MiR-506 directly targets YAP in breast cancer cells

In order to investigate the mechanism of miR-506 in the development of breast cancer, we screened the target genes of miR-506 using microRNA.org and Targetscan (http://www.microrna.org and http://www.targetscan.org/). YAP was identified as a candidate, as it has been consistently reported to be oncogene in various cancers. As shown in Figure 8, we found that YAP was over-expressed in all 12 breast cancer tissue compared to adjacent normal specimens (P<0.05). Figure 9A shows the potential binding sites of miR-506 to YAP 3'-UTR according to prediction in the website of microRNA.org, and recently one paper had already reported that YAP is a direct target of miR-506 in hepatoma cells [7]. To confirm that miR-506 can bind to the predicted site, we performed a luciferase reporter assay in the 293T cell line. As shown in Figure 9B, the luciferase activity significantly decreased after co-transfection with psi-CHECK-2/YAP 3'-UTR and miR-506 mimics in comparison with control cells, indicating that miR-506 specifically binds to the 3'-UTR of YAP mRNA. The impact of miR-506 transfection on YAP protein expression in MDA-MB-231 cell line was respectively assessed using Western blot assays (Figure 10). We found that YAP expression at the protein levels obviously decreased in the miR-506 mimics-treatment group relative to NC group and miR-506 inhibitor group. These results support our previous hypothesis.

Discussion

The discovery of the first miRNA, lin-4, in Caenorhabditis elegans initiated a new era of miRNA biology. Since then, thousands of miRNAs have been identified and annotated. Furthermore, growing evidence indicates that miRNAs may contribute to cancer pathogene-
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Figure 7. Cell cycle distribution. A. Cell cycle distribution was analyzed by flow cytometry 36 h after transfection of MDA-MB-231 breast cancer cells with 100 nmol/l miR-506 mimics or inhibitor or miR-NC. B. The respective proportion of G0/G1 phase, S-phase and G2/M phase of miR-506 mimics, miR-506 inhibitor and miR-NC groups, *P<0.05.
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Dysregulation of miRNAs is connected with initiation and progression of breast cancer, since they may serve as oncogenes or tumor suppressors [18, 19]. In the present study, we are interested in the role of miR-506 in breast cancer. We performed qRT-PCR to assess the expression level of miR-506 in breast cancer tissues and cell lines. We also researched the biological impact of miR-506 and explored the molecular mechanisms by which miR-506 modulates the behavior of breast cancer cells.

We first investigated the expression levels of miR-506 in 12-paired clinical breast cancer specimens and adjacent non-tumor breast tissues. Interestingly, we observed that the expression levels of miR-506 were significantly decreased in all 12 breast cancer samples compared to paired non-tumor tissues. In addition, we demonstrated that miR-506 expression was reduced in MDA-MB-231 cell line and MCF-7 cell line when compared with the MCF-10A non-malignant breast epithelial cell line. Similar findings were reported in many different tumors, including hepatocellular cancer [7], cervical cancer [8], gastric cancer [20], as well as in colon cancer cell lines. These data indicate that the dysregulation of miR-506 might be a common occurrence in human cancer tissue and cell lines. Further research is warranted to investigate the functional role and detailed mechanism of miR-506 in breast cancer.

To better understand the effect of miR-506 in breast cancer, we further examined the gain-or-loss of function effects of miR-506 on various aspects of breast cancer biology. MiR-506 mimics and miR-506 inhibitor were transfected into MDA-MB-231 cells to mediate its expression. The exogenous overexpression of miR-506 regulating by miR-506 mimics significantly inhibited proliferation and colony formation ability of MDA-MB-231 cells as measured by MTT and colony formation assays, respectively. Moreover, cell migration and invasion ability was also significantly reduced by overexpression of miR-506 in the MDA-MB-231 cells. We also found that miR-506 distinctly arrests cancer cells at the G1 phase when compared with the cell cycle of NC group. Previous studies demonstrated that upregulation of miR-506 might also inhibit clonogenicity and invasion in gastric cancer and clear cell renal carcinoma cell lines [20, 21]. Wang et al identified that miR-506 was down-regulated in cervical tissues and inhibited cell migration and invasion via targeting transcription factor SP1 [22]. An recent study demonstrated that miR-506 induced cell cycle arrest at the G1/S transition and enhanced apoptosis of cervical cancer lines by targeting Gli3 [8]. Another study demonstrated that miR-506 was down-regulated in breast cancer tissues and confirmed that miR-506 regulates breast cancer epithelial-mesenchymal transition by targeting Vimentin, Snai2, and CD151, which was consistent with our results [6]. Taken together, these results indi-
Dysregulated expression of miR-506 may function as a tumor suppressor in multiple cancers.

To ascertain why miR-506 exhibited these effects on the cell function, we investigated putative targets of miR-506 and identified YAP, which is the most critical effector of the Hippo pathway. In previous study, miR-506 is reported to act as a tumor suppressor by targeting YAP mRNA in liver cancer [7]. It is reported that YAP promotes breast cancer cell proliferation and survival [16, 23]. In clinical studies, positive YAP expression was suggested to be associated with shorter survival in HER2-positive breast patients [24]. In the present study, we identified YAP as a direct target of miR-506 in MDA-MB-231 cells. Furthermore, endogenous YAP expression, both at the mRNA and protein levels, was decreased in MDA-MB-231 cells transfected with miR-506 mimics, but increased when transfected with miR-506 inhibitors. In a word, these data indicate that miR-506 directly interacts with YAP mRNA and suppresses YAP protein expression.

In summary, our findings demonstrated that miR-506 is down-regulated in breast cancer tissues and cell lines, and is able to suppress cellular proliferation, migration and invasion as well as disrupt the cell cycle via direct regulation of the expression of YAP, indicating that miR-506 can serve as a potential therapeutic target for breast cancer.

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Disclosure of conflict of interest

None.

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