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Effect of miR-34a on resistance to sunitinib in breast cancer by regulating the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the influence of micro ribonucleic acid (miR)-34a on resistance to sunitinib in breast cancer, and to explore its possible underlying mechanism.

MATERIALS AND METHODS: Breast cancer MCF-7 cells were transfected with miR-34a inhibitor or mimics to downregulate or upregulate the expression of miR-34a. Then, the transfected cells were treated with sunitinib. Next, transwell assay was applied to detect the changes in cell invasion ability. Cell viability was measured *via* cell counting kit-8 (CCK8) assay. Dual-Luciferase reporter gene assay was employed to determine the interaction between miR-34a and the Wnt/β-catenin signaling pathway. The immunoblotting assay was used to measure the expression changes of proteins in the pathway.

RESULTS: The overexpression of miR-34a significantly reduced the invasive ability of MCF-7 cells after treatment with sunitinib. After miR-34a expression was downregulated, the sensitivity of MCF-7 cells to sunitinib was significantly lowered. MiR-34a interacted with the 3'-untranslated region (3'-UTR) on Wnt1. Meanwhile, the overexpression of miR-34a remarkably downregulated the messenger RNA (mRNA) and the protein levels of Wnt1, whereas upregulated the expressions of Wnt1 and β-catenin.

CONCLUSIONS: MiR-34a affects the sensitivity to sunitinib in breast cancer by regulating the Wnt/β-catenin signaling pathway.

Key Words
MiR-34a, Wnt/β-catenin, Sunitinib, Breast cancer, Drug resistance.

Introduction

Breast cancer, a common malignant tumor, is the second leading cause of cancer-related deaths in females[1]. Surgery and postoperative adjuvant chemotherapy are the main treatment methods for breast cancer. However, due to the adverse side effects of chemotherapy and drug resistance, the prognosis of most breast cancer patients remains poor[2]. Anti-angiogenic therapy interfering with vascular endothelial growth factor (VEGF) or its receptor (VEGFR) has shown promising results in the treatment of breast cancer. Sunitinib is an effective multi-target receptor tyrosine kinase inhibitor (TKI), which has an evident inhibitory effect on VEGFR. It is reported that sunitinib has been applied in clinical trials of advanced breast cancer. However, the efficacy of sunitinib alone or in combination with other chemotherapeutic drugs is mostly unsatisfactory.

Recently, multiple evidence has proved that micro ribonucleic acids (miRNAs) are key players in cancer biology[3]. In addition, miRNAs are endogenous non-coding RNA molecules that can regulate one-third of all human protein-coding genes. They also affect various biological processes, including cell proliferation, differentiation, fate determination, apoptosis, organ damage and cancer[3]. As one of the most common miRNAs associated with the prognosis of cancer patients, miR-21 has become a novel molecular target for cancer therapy[4]. MiR-21 is an oncogene that is overexpressed in many types of malignant tumors[5]. In breast cancer, miR-21 expression is significantly increased and is correlated with poor survival of patients[6]. B-cell lymphoma 2

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(Bcl-2), which is regulated by miR-21, is a key regulator in the apoptosis of many human tumor cells. Besides, it has been found that miR-203 is overexpressed in human breast cancer. The knockdown of miR-203 upregulates suppressor of cytokine signaling 3 (SOCS3), making breast cancer MCF-7 cells sensitive to cisplatin-mediated apoptotic cell death[7]. On the contrary, miR-34a is an important tumor suppressor in many types of malignancies, including breast cancer[8]. Moreover, miR-34a regulated by the p53 network is significantly reduced in many types of human cancers[9]. Furthermore, overexpressed miR-34a in breast cancer induces cell apoptosis and inhibits cell proliferation and migration by targeting Bcl-2 and various cyclins[10]. These findings indicate that miR-21, miR-203 and miR-34a play crucial roles in the progression of cancers as oncogenes or tumor suppressor genes.

Materials and Methods

Cell Culture

Human breast cancer MCF-7 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Wuhan, China). All cells were cultured in high-glucose Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 μg/mL streptomycin and 100 IU/mL penicillin. Then, cell culture flasks were placed in an incubator with 5% CO_2 and 95% humidity at 37°C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells treated with different ways were collected. Total RNA was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, 1 μL RNA solution was used, and the concentration and purity of the extracted RNA were measured using a microplate reader. The ratio of optical density at 260 and 280 nm (OD_{260}/OD_{280}) should be 1.6-1.8. The remaining RNA solution was sub-packaged and stored at -80°C for subsequent use.

Complementary deoxyribonucleic acid (cDNA) was synthesized in accordance with the instructions of PrimeScript™ Kit (TaKaRa Bio Inc., Otsu, Shiga, Japan). Firstly, a reaction system (10 μL) was prepared using 2 μg total RNA (the volume of RNA added was calculated by the respective concentrations), 1 μL Oligo (dT) primer (50 μM), 1 μL deoxyribonucleotide triphosphate (dNTP) mixture (10 mM), and corresponding enzyme-free double-distilled water. The prepared reaction system was mixed gently, heated by a PCR instrument at 65°C for 5 min and transferred onto the ice for rapid cooling. Subsequently, 4 μL 5×PrimeScript Buffer, 1 μL PrimeScript RTase, 0.5 μL RNase inhibitor, and 4.5 μL enzyme-free water were added into the above mixture, making a 20 μL reaction system. Then, the 20 μL reaction system was mixed and heated by a PCR instrument at 42°C for 45 min and 95°C for 5 min. Next, it was transferred onto the ice for rapid cooling (that was the synthesis of single-stranded cDNA) and stored at -20°C for PCR amplification.

Cell Transfection

Cells were first seeded into 6-well plate at a density of 2×10^5/well. When the confluence was up to 40-60%, cell transfection was performed. MiR-34a overexpression plasmids were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Based on the instructions provided, the optimal transfection concentration of both cells was 100 nM. The transfection efficiency was verified *via* RT-PCR. After 48 h of culture with complete medium, the transfected cells were then collected for further analysis.

Transwell Assay

Transwell assay was applied to detect the invasive and migration ability of MCF-7 cells. The 6.5 mm transwell with 8.0 μm pore polycarbonate membrane insert (Costar, Corning Incorporated, Corning, NY, USA) was used for cell migration or invasion assay. Transfected and un-transfected cells (2×10^5) were suspended with serum-free medium and inoculated into the upper chamber of the insert. Meanwhile, the complete culture medium was added into the lower chamber. After 24 h, cells on the upper surface of the membrane were removed. However, cells under the membrane were fixed with 100% methanol at room temperature for 20 min and stained with crystal violet. Next, an optical microscope (Olympus Co., Tokyo, Japan) was used for the observation, and the number of cells was counted from 5 randomly selected fields per well. Finally, the average number of migrating or invading cells was calculated.

Dual-Luciferase Reporter Gene Assay

On the first day, cells (selected appropriately according to specific assays) were digested, inoculated into 35-mm cell culture dishes and cultured in a 37°C, 5% CO_2 incubator overnight. When the cell density reached 70%, the cells were co-transfected

with Luciferase reporter plasmids, LacZ expression plasmids, and other plasmids for 24-36 h. Next, the medium was discarded and the cells were washed with pre-cooled phosphate-buffered saline (PBS) (containing no calcium and magnesium ions). Pre-cooled harvest buffer (350 μL) was added to each dish, followed by cell lysis at 4°C or on ice for 10 min. During cell lysis, sufficient 1.5 mL micro-centrifuge tubes were prepared. Adenosine triphosphate (ATP) buffer and luciferin buffer were mixed at a ratio of 1:3.6 to prepare the reaction solution. Subsequently, the prepared mixture was dispensed to the above tubes with 100 μL per tube. An equal volume of cell lysis solution (100 μL) was sequentially added to the centrifuge tubes mentioned and mixed rapidly. Finally, the optical density (OD) values were detected using a Luminometer, and data were analyzed after plotting using the corrected read value.

Cell Viability Assay

Cells were evenly inoculated into 96-well plates at a density of 5×10^3/100 μL, followed by cultivation in an incubator. Four replicate wells were set in each group. On the next day, cells were added with freshly prepared 10 umol/L sunitinib solution to a final concentration of 0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM and 1000 μM, respectively. Then the cells were incubated for 48 h. Briefly, 10 μL 10% fresh cell counting kit-8 (CCK8) solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, during which no bubbles were produced to avoid the influence on OD values. Meanwhile, blank control wells (with only cells and CCK8 solution, no drugs) and zero wells (with medium and CCK8 solution, no cells) were set. The cells were incubated in the incubator for 1-4 h depending on the reaction extent of the different cell lines. If the color was underdeveloped, the incubation could be continued to confirm the best conditions. After the reaction, OD values at the wavelength of 450 nm were measured using a multi-function microplate reader. Tumor cell growth inhibition rate = (1 - −OD in the drug treatment group/OD in the control group) ×100. Dose-dependent growth curves were plotted, and the half maximal inhibitory concentration (IC50) values of sunitinib were calculated. The experiment was repeated three times.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS 22.0, IBM, Armonk, NY, USA) was used for all statistical analysis. The *t*-test was used to compare the differences between the two groups. The one-way ANOVA was performed to compare the differences among different groups, followed by Post Hoc Test (Least Significant Difference). The two-sided 95% confidence interval (95% CI) was used. $p<0.05$ was considered statistically significant.

Results

Effect of MiR-34a on Cell Invasion

MCF-7 cells were first transfected with miR-34a mimics and inhibitors, respectively. Then the cells were treated with 10 μmol/L sunitinib for 48 h. Subsequently, *in vitro* cytological functional tests were performed. Transwell assay was applied to detect the changes of cell invasion. Results showed that the overexpression of miR-34a significantly reduced the invasion ability of MCF-7 cells ($p<0.05$) (Figure 1A, 1B).

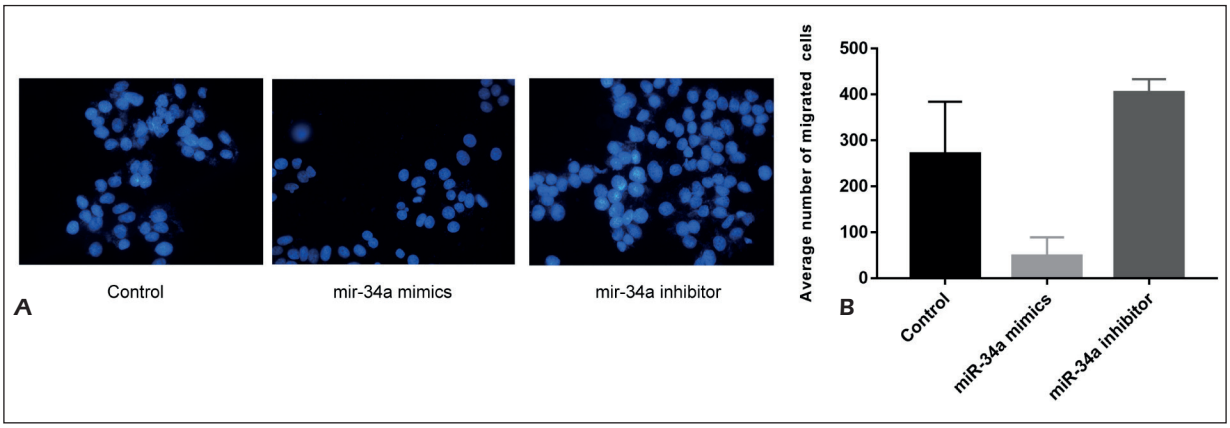

Figure 1. Effect of miR-34a on the invasion ability of cells. **A-B**, transwell assay demonstrated that the overexpression of miR-34a significantly reduced the invasion ability of MCF-7 cells ($p<0.05$).

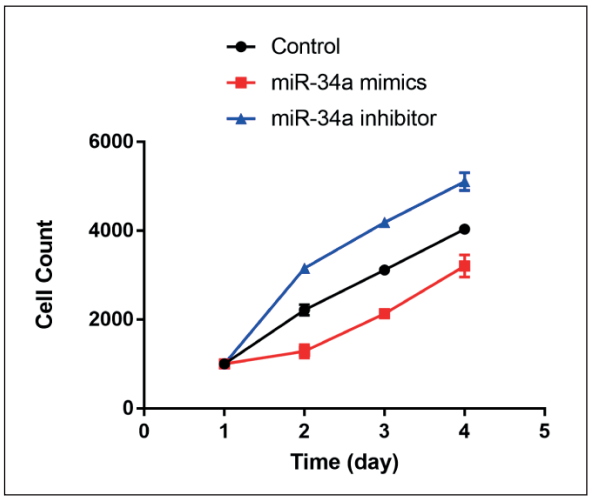

Figure 2. Cell proliferation determined by CCK-8 assay. The sensitivity of MCF-7 cells to sunitinib was significantly reduced after miR-34a downregulation ($p<0.05$).

Influence of MiR-34a on Cell Proliferation

CCK8 assay was performed to detect the proliferation of MCF-7 cells. Results demonstrated that the sensitivity of MCF-7 cells to sunitinib was remarkably reduced after miR-34a inhibition (Figure 2).

Dual-Luciferase Reporter Gene Assay

In this study, bioinformatics was used to predict the target genes of miR-34a. Results showed that the 3'-UTR of Wnt1 was highly conserved to bind to miR-34a (Figure 3A). Luciferase reporter gene assay indicated that miR-34a mimics transfection significantly inhibited relative Luciferase activity of MCF-7 cells. This indicated that miR-34a suppressed Wnt1 expression by interacting with the 3'-UTR of Wnt1 (Figure 3B).

MiR-34a Overexpression Inhibited the Wnt/β-Catenin Signal Transduction Pathway

The overexpression of miR-34a significantly downregulated the mRNA and protein expression levels Wnt1 (Figure 4A, 4B). After transfection with miR-34a mimics for 48 h, statistically significant differences were found in the expressions of Wnt1 and β-catenin among different groups. Compared with controls, the expressions of Wnt1 and β-catenin were remarkably upregulated after overexpression of miR-34a, which could be reversed by the activation of the Wnt/β-catenin pathway (Figure 4C, 4D).

Discussion

Early detection and systemic treatment of breast cancer have made gratifying progress in recent years. Due to the metastasis, recurrence and treatment failure, breast cancer is still one of the leading causes of cancer-related deaths. Generally, treatment failure resulted from multidrug resistance (MDR) is very common in tumor cells. More and more evidence has shown that MDR in breast cancer is caused by various mechanisms. Previous studies have concluded that the mechanisms of MDR in breast cancer include abnormal expressions of anti-oncogenes, such as MDR1 and breast cancer resistance protein (BCRP). In general, these genes affect the efficacy of drugs by reducing their concentrations in cells. In addition, the development of MDR also includes the expressions of anti-apoptotic genes such as Bcl-2 and p53. Moreover, multiple studies have also demonstrated that DNA methylation and histone modification are the main causes of MDR in breast cancer[11].

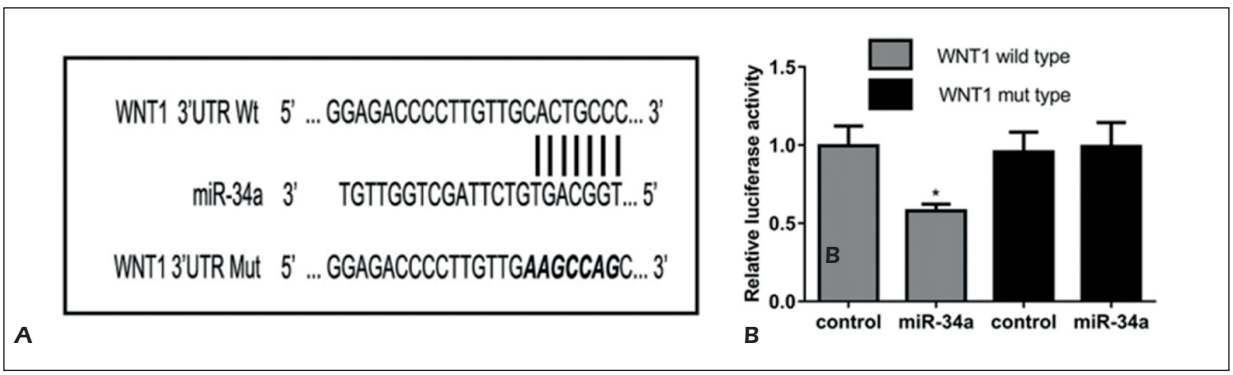

Figure 3. MiR-34a directly targeted to the 3'-UTR of Wnt1. **A**, Predicted binding site of miR-34a to the 3'-UTR in Wnt1. **B**, Luciferase reporter gene assay indicated that miR-34a suppressed WNT1 expression by interacting with Wnt1.

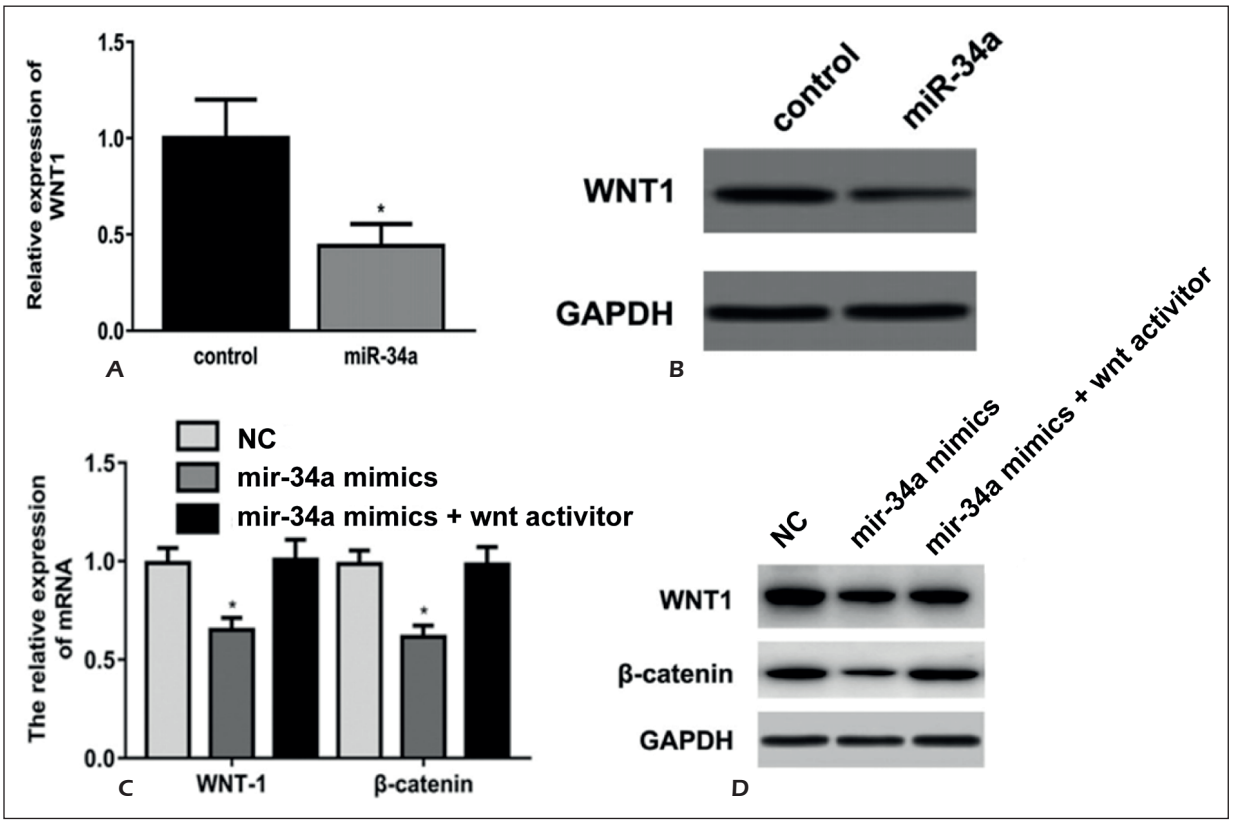

Figure 4. Interaction between miR-34a and Wnt1. **A-B**, MiR-34a overexpression significantly in-hibited the expression of Wnt1. **C-D**, The expressions of Wnt1 and β-catenin were increased after transfection with miR-34a mimics, which could re-versed by the activation of the Wnt/β-catenin pathway.

As for the treatment of breast cancer, anti-angiogenic therapy has exerted satisfying results by blocking VEGF or VEGFR[12,13]. Sunitinib is an efficient multi-target receptor TKI that can significantly suppress VEGFR. However, the response of some patients to sunitinib is unsatisfactory[14,15]. Researchers have found that sunitinib increases the number of human cancer stem cells, thus limiting its effectiveness. In this study, we found that the resistance to sunitinib might be related to the abnormal activation of the Wnt/β-catenin signaling pathway.

Besides, it was discovered in this study for the first time that miR-34a acted on the Wnt/β-catenin signaling pathway, thereby impacting the sensitivity to sunitinib. Gene encoding miR-34a is located on lp36.23, which is one of the first widely studied miRNAs relating to tumorigenesis. It has been reported[16] that miR-34a inhibits the proliferation of breast cancer cells by suppressing the Akt signaling pathway. The recovery of miR-34a regulates Bcl-2, eventually reducing the proliferation and migration of breast cancer[17]. Moreover, p53-regulated miR-34a can repress the invasion and metastasis of breast cancer[18].

MiR-34a was originally identified as a target gene for p53. Previous studies have shown that miR-34a is silenced in various human cancers, and acts as an important tumor suppressor. Increasing evidence has manifested that miR-34a can antagonize multiple different carcinogenic processes, such as inhibiting the differentiation, proliferation, migration, and invasion of tumor cells, as well as increasing cell apoptosis and arrest[19]. Moreover, it has been reported that down-regulation of miR-34a in different types of cancer is associated with MDR. Some studies have demonstrated that increased expression of miR-34a in cells can significantly enhance the sensitivity of cancer cells to cisplatin. However, decreased expression of miR-34a leads to drug resistance of colorectal cancer DLD-1 cells to 5-FU. Oxaliplatin induces the downregulation of miR-34a and increases drug resistance by activating macro-autophagy in CRC cells[20].

Kojima et al[21] have reported that decreased or lost expression of miR-34a in pancreatic cancer PC3PR cells elevates the expressions of silent information regulator 1 and Bcl-2, eventually resulting in resistance to taxanes. In this study, results found that MCF-7 cells had a significantly lower sensitivity to sunitinib after down-regulating miR-34a. This suggested that miR-34a might be involved in the MDR process of breast cancer. Further in-depth studies and cell rescue experiments revealed that the effect of miR-34a on the sensitivity to sunitinib in breast cancer was achieved by acting on the Wnt/β-catenin signaling pathway. As a central promoter of the signaling pathway, the transport of β-catenin to the nucleus leads to the expressions of growth and invasion-related genes, including cyclin D1 and cMyc. In this study, bioinformatics analysis predicted that miR-34a tightly targeted to the 3'-UTR of Wnt1. Also, overexpressed miR-34a significantly reduced the mRNA and protein production of Wnt1. These results confirmed that miR-34a affected the sensitivity to sunitinib in breast cancer by down- and up-regulating the synthesis of miR-34a. Our study might provide new insights on miR-34a in the development and treatment of breast cancer.

Conclusions

We demonstrated that the miR-34a affected the sensitivity of sunitinib in breast cancer by regulating the Wnt/β-catenin signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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