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MiR-216a-5p act as a tumor suppressor, regulating the cell proliferation and metastasis by targeting PAK2 in breast cancer

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Abstract. – OBJECTIVE: Our study aimed to investigate the expression of microRNA-216a-5p (miR-216a-5p) in breast cancer (BC) and its effect on the proliferation and metastasis of BC cells by regulating the expression of p21-activated protein kinase 2 (PAK2) gene.

PATIENTS AND METHODS: A total of 50 cases of cancer tissue specimens and corresponding para-carcinoma normal tissue specimens were collected from the breast surgery department of our hospital from July 2016 to December 2017. BC MCF-7 cell line and normal breast epithelial MCF-10A cells were cultured. MiR-NC (negative control), LV-p21-activated protein kinase 2 (PAK2) and/or miR-216a-5p mimics were synthesized and transfected. The protein and mRNA expression level in BC tissues and cells were detected by Western blot and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay, respectively. Additionally, the Luciferase Reporter Assays, cell proliferation detection, clone formation assays and transwell migration and invasion assay were performed to determine the functional alteration of BC cells, respectively.

RESULTS: The results of qRT-PCR demonstrated that miR-216a-5p was decreased in both BC tissues and cells compared with that in normal controls. Online target gene prediction software and Dual-Luciferase reporter assay were used for target identification, and PAK2 was identified as a functional target of miR-216a-5p in BC cells. The results were further clarified with the Western blot (WB) experiment. *In vitro*, cell functions were detected by Cell Counting Kit-8 (CCK-8), crystal violet staining and transwell experiment, respectively. The results indicated that decreased expression of PAK2 resulting from the up-regulation of miR-216a-5p could restrain the proliferation, clone formation, invasion and migration abilities of BC cells.

CONCLUSIONS: We showed that miR-216a-5p played a role as antioncogene in BC, which provides a new therapeutic target for the treatment of BC.

Key Words:
Breast cancer, MicroRNA-216a-5p (miR-216a-5p), P21-activated protein kinase 2 (PAK2), Proliferation, Invasion and migration.

Introduction

Breast cancer (BC) is a common malignancy in women. There are approximately 1.5 million new cases of BC worldwide each year, and its incidence rate is the highest among those of all categories of female malignancies[1,2]. The number of patients dying from BC is as large as 0.57 million each year, accounting for 14% of all malignancy-related deaths among women[3,4]. The clinical statistical data showed that the incidence of BC in China is increasing at an annual speed of 2-3%. Currently, BC had been ranked among the malignancies with the fastest rise in the incidence rate, which was second only to lung cancer. Moreover, BC was more likely to attack young women[5]. BC poses a serious threat to the life and health of women, so seeking effective prevention and treatment methods is of great significance. With the continued development of modern medicine and the improvement of medical technologies, the mortality rate of clinical

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BC patients has been decreased by 34%, and the 5-year survival rate has risen to 90%. However, the death rate of BC patients remained high and ranked second among all categories of malignant tumors in women[6]. The metastasis feature of BC was considered the primary cause of the relapse and poor prognosis in patients. According to the clinical information, about 10-15% of BC patients exhibited a distant metastasis within 3 years after being definitely diagnosed, and the 5-year survival rate of those with metastatic BC was only 23%[7,8]. Currently, surgical resection is still the major method of clinical treatment for BC[9,10]. However, BC is characterized by the latent onset and usually exhibited non-typical clinical symptoms in most cases. Thus, there is a scarcity of effective and reliable screening methods for BC now. Meanwhile, due to the fast progression of BC, most patients are in the advanced stage when they go to the hospital, thus missing the best time for surgeries and effective interventions. Therefore, we emphasized the importance of finding effective molecular markers for the diagnosis and treatment of BC. More and more evidence has indicated that micro-ribonucleic acid (miRNA) was correlated with the occurrence and development of malignant tumors, such as BC[11-13]. MiRNA is a class of small non-coding single-stranded RNA molecules with 20-24 nt in length, and widely exists in animals and plants. MiRNAs exert a regulatory effect on the expressions of the specific genes by binding to the target gene in the form of incomplete complementary pairing[14]. Up to now, although the miRNAs discovered in human cells accounts for no more than 2% of human genomes, they have been reported to affect the expression of nearly 1/3 genes and play regulatory roles in a series of physiological processes in human body[15,16]. Moreover, the miRNA-related genes also served as key players in the growth, proliferation, cycle and apoptosis of cells[17]. MiR-216a-5p, as one of the members of miRNA family, participated in the development of various diseases. Menghini et al[18] found that miR-216a-5p controlled autophagy in human umbilical vein endothelial cells by regulating the expression of Beclin1. In a rat model of L-arginine-induced acute pancreatitis, the plasma concentration of miR-216a-5p was found significantly increased. Endo et al[19] speculated that miR-216a-5p might be a biomarker for acute pancreatitis injury. In bone metabolic diseases, miR-216a-5p could promote the osteoblast differentiation and enhance the bone formation[20]. Besides, in the study of malignant tumors, miR-216a-5p was commonly found as a tumor suppressor that inhibited the malignant effects[21-23]. However, the study of miR-216a-5p in BC has not been reported yet.

Patients and Methods

Patients

A total of 50 cases of cancer tissue specimens and corresponding para-carcinoma normal tissue specimens were collected from the Breast Surgery Department of our hospital from July 2016 to December 2017. All specimens were collected from the diagnosed patients, who received no radiotherapy or chemotherapy before the operation. Para-carcinoma normal tissue specimens were taken more than 5 cm away from cancer tissues. All tissue specimens were quickly cryopreserved in liquid nitrogen after resection. This study was approved by the Ethics Committee of West China Hospital. Signed informed consents were obtained from all participants before the study.

BC MCF-7 cell line and normal breast epithelial MCF-10A cells were purchased from Shanghai Bioleaf Biotechnology Co., Ltd. (Shanghai, China) and cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) under 5% CO_2 at 37°C. The cells in the logarithmic growth period were used for experiments.

Transfection

MiR-NC (negative control), LV-p21-activated protein kinase 2 (PAK2) and/or miR-216a-5p mimics were synthesized by RiboBio (Guangzhou, China) and transfected into MCF-7 cells by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the instructions. About 6 h later, the cells were continued to be cultured in the new Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) for subsequent experiments.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted *via* TRIzol method (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribonucleic acid (cDNA). Then, with the cDNA as the template, qPCR was conducted

to detect the expressions of miR-216a-5p. The experiment results were calculated by the $2^{-\Delta\Delta Ct}$ method. U6 was selected as the internal reference of miR-216a-5p. Primer sequences used in this study were as follows: miR-216a-5p, F: 5'-ACATCCTCGGCCAGTAAGACTG-3', R: 5'-GTCGACCAGATTGCGTTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Luciferase Reporter Assays

The 3'-untranslated region (UTR) (Wt-PAK2-3'-UTR) of the wild-type PAK2 and the 3'-UTR of the mutant-type PAK2 (Mut-PAK2-3'-UTR) were co-transfected into MCF-7 cells with the empty plasmid and miR-216a-5p overexpression plasmid for 48 h. The Luciferase activity was detected using the Dual-Luciferase reporter gene assay kit. Then the cells were washed with Phosphate-Buffered Saline (PBS) 3 times, lysed with PLB, and shaken on a shaking table for 30 min. The cell lysis solution was mixed and blown evenly with LARII. The intensity of Firefly Luciferase reaction was measured, and the intensity of Renilla Luciferase reaction was also measured after Stop & Glo Reagent was added (Solarbio, Beijing, China).

Western Blot (WB) Analysis

After being transfected for 48 h, the cells were collected and lysed to extract the protein, followed by addition of loading buffer and boiling at 100°C for 10 min. Then, the protein was isolated *via* 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After that, the membranes were blocked with Tris-Buffered Saline with Tween-20 (TBST) containing 50 g/L of skimmed milk at room temperature for 1 h. The membranes were then incubated with the corresponding rabbit anti-human PKA2 antibody and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody at 4°C overnight. On the next day, the membranes were washed with TBST 3 times and incubated with horseradish peroxidase (HRP) to mark the goat anti-rabbit IgG antibody for 1 h at room temperature. These protein samples were washed with TBST 3 times, fixed and developed via enhanced chemiluminescence (ECL) development system (Thermo Fisher Scientific, Waltham, MA, USA). The expression level of the protein was quantified with GAPDH as the internal reference.

Cell Proliferation

The transfected cells in each group were inoculated into a 96-well plate at 4×10^3 cells/well, and the proliferation ability of the cells was detected on d1, d2, d3, d4. The specific operation is as follows: each well was added with 15 μL of Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) at each time point and placed in an incubator for 4 h. The absorbency value (A) at 450 nm of each well was then determined to plot the growth curve.

Clone Formation Assays

At 48 h after the transfection, the cells were collected and inoculated into a new 6-well plate at 1×10^3 cells/well. 4 repeated wells were set in each group. After 10 d of culture, the cells were fixed in anhydrous alcohol for 15 min and stained with 0.1% crystal violet for 10 min. The cells were then washed with PBS buffer 3 times (5 min/time) and subjected to photography and counting.

Transwell Migration and Invasion Assay

The cells were digested, added with the serum-free DMEM and evenly mixed by the blowing method. After the cell concentration was adjusted to 5×10^5 cells/mL, the lower chamber of transwell was added with 800 μL of DMEM containing 5% FBS and the upper chamber with 200 μL of cell suspension. Before the experiment, the stored liquid Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was diluted with the serum-free DMEM and antibiotics (1:6) and added into transwell at 50 μL/well. The cells were then cultured in an incubator for 36-48 h, followed by 20 min of fixation with 4% paraformaldehyde and 20 min of staining with crystal violet. The cells on the membrane were carefully wiped off using cotton swabs. Finally, the system for microscopic image acquisition was used to observe the migration and invasion of cells and visual fields were randomly selected and photographed.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Independent-samples *t*-test was used for the comparison between the two groups. One-way analysis of variance was adopted for the comparison among groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Abnormal Expression of MiR-216a-5p in Clinical Cases and Cells

The expression level of miR-216a-5p was measured using the qRT-PCR assay. The results showed that, whether in tissues or cell lines, miR-216a-5p manifested a significant decrease. The expression of miR-216a-5p in BC tissues and BC cell lines was nearly 80% and 60% lower than that of para-carcinoma normal tissues and normal epithelial cell lines, respectively ($p<0.001$) (Figure 1).

Detection of Transfection Efficiency

The transfection efficiency was confirmed by qRT-PCR assay. As shown in Figure 2B, transfection of miR-216a-5p mimic markedly increased the expression of miR-216a-5p in MCF-7 cells ($p<0.01$), whereas miR-NC had no effect on the expression of miR-216a-5p ($p>0.05$).

PAK2 is a Direct Target of MiR-216a-5p

Using the online target prediction tool, we found that miR-216a-5p had complementary sequences with the 3'-UTR interval of PKA2 (Figure 2A). Based on the role of PKA2 in recent cancer studies, we further explored the potential conjugation between miR-216a-5p/PKA2 and BC. The Luciferase Reporter results clarified the targeted regulation of miR-216a-5p on PAK2. When compared with the NC group, the Luciferase activity in MCF-7 cells was significantly decreased after being co-transfected with miR-216a-5p mimics and pmirGLO-PAK2 3' UTR (wt) plasmids. However, the Luciferase activity showed no alteration when the pmirGLO-PAK2 3' UTR (mut) plasmid was transfected (Figure 2C).

It has been reported that miRNAs usually function by regulating the expression of the target gene after transcription[14]. Therefore, we examined the protein expression of PKA2 by Western blotting assay. After transfection of miR-216a-5p, PAK2 was markedly downregulated (Figure 3D). All the above results suggested that miR-216a-5p had a clear regulation of the expression of PAK2 in MCF-7 cells. However, further exploration is necessary for the role of miR-216a-5p on MCF-7 cell functions.

MiR-216a-5p Inhibited the Cell Proliferation

The acquisition of non-inhibitory proliferation is an important feature of cancer cells. In our experiments, we first observed the changes in the proliferative capacity of MCF-7 cells. We planted MCF-7 cells transfected with miR-216a-5p mimic into 96-well plates and detected the cell viability via the CCK8 assay. As shown in Figure 3A, the cells in the miR-NC group showed exponential growth, while the proliferation curve of the cells in the miR-216a-5p transfection group was remarkably suppressed. There was a significant difference in cell viability between the control group and the treatment group on the fourth day after transfection ($p<0.01$).

In the clone formation experiment, the cell proliferation inhibition of miR-216a-5p was simultaneously demonstrated. The overexpression of miR-216a-5p significantly inhibited the clonal clone formation of MCF-7 cells ($p<0.001$) (Figure 3B-3C). Besides, the migration and in-

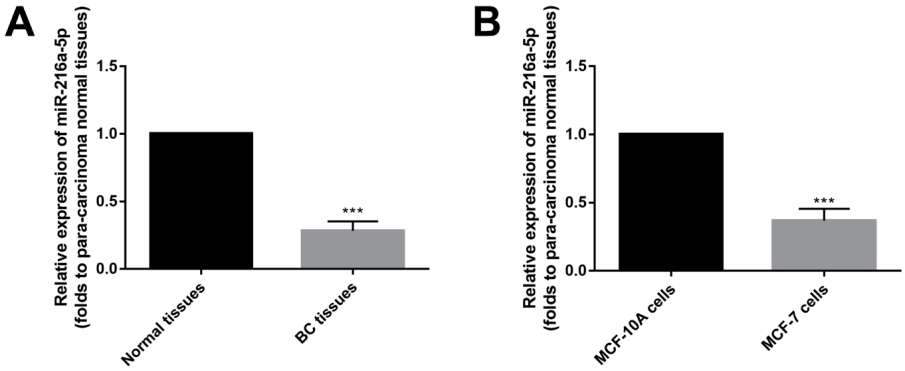

Figure 1. The expression of miR-216a-5p in BC tissue samples and BC cell. ***A,*** Difference in the expression of miR-216a-5p between BC tissues and corresponding adjacent normal tissues (***$p<0.001$). ***B,*** The expression of miR-216a-5p in BC MCF-7 cell lines and normal breast epithelial MCF-10A cells (***$p<0.001$).

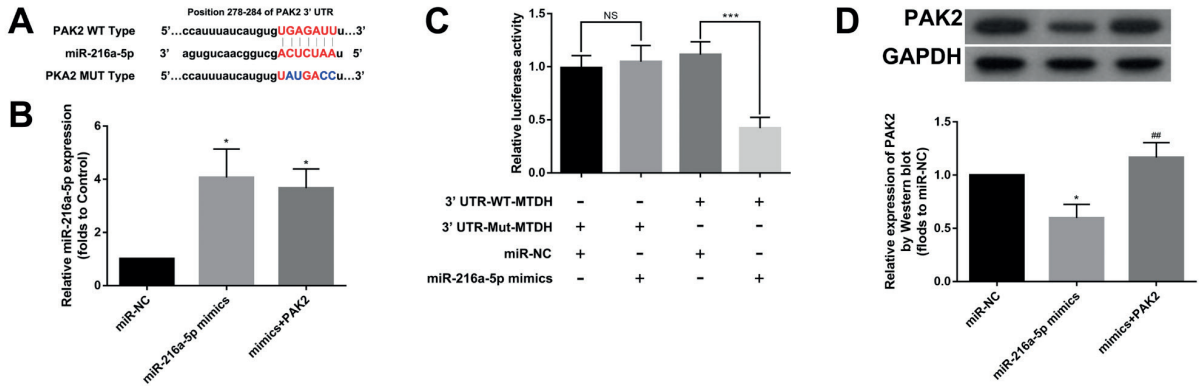

Figure 2. PAK2 is a direct and functional target of miR-216a-5p. ***A,*** Diagram of putative miR-216a-5p binding sites in PAK2 mRNA. ***B,*** Transfection efficiency detected by qRT-PCR. (*p<0.05). ***C,*** Relative activities of Luciferase reporters (***p<0.001). ***D,*** The expression of PAK2 in BC cells. Data were presented as means ± standard deviations (**p<0.01 *vs.* NC group; #p<0.05 *vs.* Mimics group).

vasion to surrounding tissues is an important mode for the spread of cancer cells and also reflects the ability of metastasis of cancer cells. In the transwell experiment, the number of invasive and migratory cells were much less in the miR-216a-5p group than those in the miR-NC group (p<0.01) (Figure 4). However, in the co-transfection group of miR-216a-5p and PAK2, the proliferation and metastasis ability of MCF-7 cells was restored (p>0.05), suggesting that the inhibition of PAK2 could ameliorate the malignant behavior of MCF-7 cells.

Discussion

BC is one of the malignant tumors that pose a great threat to woman's life and health worldwide. Finding effective detection and treatment methods is the current focus and difficulty. The development of BC was attributed to the collective effect of a series of factors *in vivo* and *in vitro*, including the increase in the expression level of oncogenes and (or) the decreased expression of cancer suppressor genes. As a category of small non-coding single-stranded RNA molecules with high evolutionary conservation, MiRNAs are ubiquitous in all eukaryotes. Single miRNA could directly regulate the expression of a certain gene or hundreds of genes, and the expression of the same gene can also be affected by multiple microRNAs at the same time. The se miRNA-mRNA interactions form various complex regulatory networks. Therefore, it is feasible to dig into the correlation of miRNA with BC, and to diagnose and judge the prognosis of BC based on the abnormal expression characteristics of miRNA in BC. It has been reported that miRNAs post-transcriptionally down-regulate the expression of the target genes, and thus function in the malignant process of tumors. To understand the mechanism of miR-216a-5p in promoting the growth of BC cells, it was vital to identify its target gene. Simultaneously, PCR and WB assay were performed to determine the corresponding relationship between miRNA and target gene by detecting the

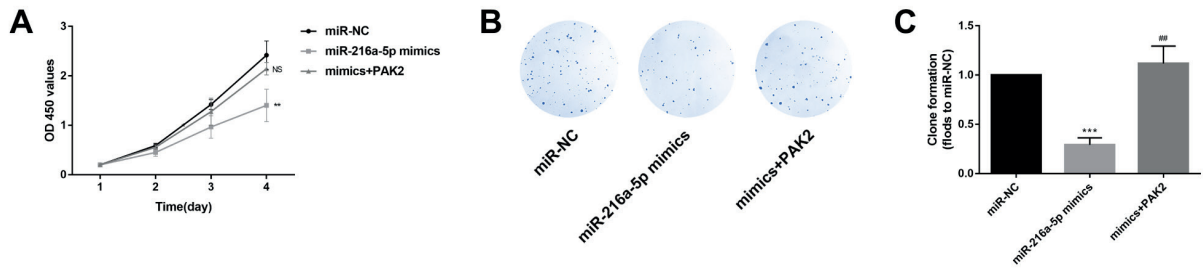

Figure 3. ***A,*** MiR-216a-5p inhibited the proliferation of BC cell (**p<0.01 *vs.* NC group). ***B-C,*** Assessment of colony formation (***p<0.001 *vs.* NC group; ##p<0.01 *vs.* Mimics group). All data were presented as means ± standard deviations.

expression levels of mRNA or protein of the target gene.

In this work, miRanda and Targetscan databases were used for the prediction of target genes of miR-216a-5p. It was discovered that miR-216a-5p and PAK2 had binding sequences. PAK2 as a class of serine/threonine protein kinase with high and strict sequence homology, had attracted our attention due to the studies on its role in tumors over the recent years. Previous studies had corroborated that PAK2 could participate in multiple intracellular biological activities, such as cytoskeletal reorganization and cell movement, *via* various pathways[24-26]. Additionally, PAK2 could be activated by numerous upstream signals, especially the Rac of the G protein Rho family and cell division cycle 42 (CDC42), and also regulated several important signaling pathways and cell functions[24,27]. Furthermore, recent findings had indicated that PAK2 activation was closely related to the extent and prognosis of malignant tumors including melanoma[28], ovarian cancer[29] and gastric cancer[30].

Subsequently, the fluorescein enzyme reporter system demonstrated that the down-regulation of miR-216a-5p markedly raised the expression level of the Luciferase in MCF-7 cells transfected with the wild-type PAK2 expression vector, while no significant change was observed in the MCF-7 cells transfected with the mutant-type PAK2 expression vector. The above results demonstrated that PAK2 was the target gene of miR-216a-5p. Afterward, to further validate this conclusion, WB experiment was performed. The results showed that, compared with those in the miR-NC group, the expression levels of PAK2 protein in miR-216a-5p mimics group was remarkably decreased. The above results showed that PAK2 was the target gene of miR-216a-5p. *In vitro*, we analyzed the effects of miR-216a-5p on BC cell functions and found that the up-regulation of miR-216a-5p inhibited cell proliferation and reduced the migration and invasive abilities of BC cells. However, the overexpression of PAK2 could effectively reverse the aforementioned phenomenon induced by miR-216a-5p, suggesting that the high expression of miR-216a-5p in BC might limit the abnormal cell proliferation and metastasis capacities by targeting PAK2.

Conclusions

We provided evidence for the ability of miR-216a-5p to regulate the proliferation and metastasis of BC cells, which offered a new theoretical basis for further studies on BC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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