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ORIGINAL ARTICLE

Effect of miR-200c on the proliferation, migration and invasion of breast cancer cells and relevant mechanisms

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Summary

Purpose: *The current study aimed to explore the effect of miR-200c on the proliferation, migration and invasion of breast cancer cells and its relevant mechanisms.*

Methods: *Cell counting kit-8 (CCK-8), scratch wound healing assay and Transwell assay were performed after upregulation of miR-200c to detect the capabilities of proliferation, migration and invasion of MCF-7 breast cancer cells. Also, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot were carried out to determine the expression levels of fucosyltransferase-4 (FUT4) and relevant genes in PI3K/AKT signaling pathways.*

Results: *miR-200c upregulation in MCF-7 cells decreased the capabilities of proliferation, migration and invasion in MCF-7 cells. MiR-200c could regulate the level of FUT4 in MCF-7 cells, and might affect the cell proliferation, migration and invasion through PI3K/AKT signaling pathway.*

Conclusions: *The results of this study indicated that miR-200c might serve as a new target in the diagnosis and treatment of breast cancer. MiR-200c regulated the expression of FUT4, and affected the biological behaviors of breast cancer MCF-7 cells, such as proliferation, migration and invasion.*

Key words: *breast cancer, FUT4, miR-200c, PI3K/Akt*

Introduction

Breast cancer is a common malignant tumor in females. In China, there are about 130,000 new cases of breast cancers in females and 40,000 patients die of breast cancer every year. According to estimations, there will be an increasing tendency in both the incidence and mortality rates in breast cancer in the future [1]. Currently, the mechanisms of occurrence and development of breast cancer remain unclear. The pathogenesis of breast cancer includes genetic factors and gene mutations. Some of breast cancers have been caused by genetic factors, i.e. the younger the patient, the higher the genetic tendency [2]. Multiple oncogenes have been confirmed to be involved in the occurrence and progression of breast cancer as well, and the abnormality in oncogenes can induce mutations in cells, contribute to the initiation and progression of breast cancer. The mortality rate of breast cancer ranks 1st among the malignant tumors in females. Thus, the research into the diagnosis and pathogenesis of breast cancer is quite urgent. Specific molecular mechanisms in the diagnosis, initiation and progression of breast cancer are expected to be identified providing a reliable reference for the clinical disease diagnosis and treatment.

Fucose, an hexose, includes two kinds of configurations, i.e. the L-fucose and D-fucose. Glycoproteins and glycolipids spread on the surface of eukaryotic cells contain the fucosylated carbohydrate chain. The residue of fucose is also involved

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in the cell growth, adhesion and migration [3]. In addition, fucose participates in the synthesis of various carbohydrate chains, and abnormal fucolysation variations have been found in the occurrence and progression of various tumors, such as breast cancer and liver cancer [4].

Glycosylation, with a great significance in biology, is a kind of process and modification after protein translation. In the initiation and progression of breast cancer, alterations may occur in the glycosylation of surface proteins expressed in different stages of breast cancer, and are correlated with poor prognosis. The occurrence and development of tumor are always accompanied by abnormal fucosylation that may be caused by the metabolism of tumor cells. Fucosylation also participates in the tumor biological behaviors, such as growth, metastasis and infiltration of tumor cells. Research has found that fucose exists widely on the membrane of breast cancer cells with high metastatic capability. Decrease of fucose residue on the cell surface significantly decreases the metastatic capability of breast cancer cells.

FUT4 is a a-1,3-fucosyltransferase. Some studies have found that a-1,3-fucosyltransferase is associated with the proliferation [5] and apoptosis [6] of tumor cells. In many kinds of tumors (such as breast cancer, gastric cancer and ovarian cancer), the greatly increased serum activity of a-1,3-fucosyltransferase could reverse to normal after tumor treatment, indicating that the activity of a-1,3-fucosyltransferase is correlated with the tumor size and metastasis [6]. In about 70-90% of the epithelial tumors, such as breast cancer, high expression of Lewis Y (LeY) antigen that is synthesized under the catalytic effect of FUT4 is in close correlation with metastasis, invasion and grade of tumor malignancy. Thus, it can serve as an indicator for early tumor diagnosis and prognosis [7].

MicroRNAs (miRs), a kind of small, non-coding and endogenous RNAs of 18-25 bp in length, can degrade mRNA or inhibit its translation to regulate the gene expression through binding with 3'UTR of mRNA [8]. Thus, they can participate in the regulation of various important biological processes, including proliferation, apoptosis and metabolism of cells. Dysregulation of miRs has been found in many diseases, like tumors [9]. The family of miR-200 is an important member of miRs, consisting of miR-200a, miR-200b, miR-200c, miR-141 and miR-429. Relative studies have revealed that miR-200 family can participate in epithelial-mesenchymal transition (EMT), cell senescence, survival and other key processes, indicating its critical role in diseases and embryonic development. Analysis through bioinformatics has indicated that there are thousands of target genes of miR-200c and miR-200c can participate in the pathogenesis of tumor through acting on the target genes.

We found that FUT4 is one of candidate target genes of miR-200c. A previous study [10] has confirmed the high expression of FUT4 in breast cancer cells could promote the EMT, which is positively correlated with the potential of proliferation and metastasis of breast cancer cells. However, the correlation between miRs and FUT4 has not been reported yet in the literature. MiR-200c is expressed lowly in breast cancer cells with high malignant potential, but highly in cells with low malignant potential. So, whether miR-200c could exert its effect on the occurrence and development of breast cancer through acting on FUT4 remains unknown, and the relevant functions and mechanisms remain to be further explored.

Methods

Cell and reagent

Human breast cancer cell MCF-7 was purchased from ATCC Cell Bank (Manassas, VA, USA). MCF-7 cells were cultured in the DMEM/F12 medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 μg/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in an incubator with 5% CO_2, 37°C and 90% humidity. Lipofectamine 2000, the transfection reagent, was purchased from Invitrogen (Carlsbad, CA, USA); miR-200c mimics was obtained from GenePharma (Shanghai, China) and reverse transcriptase-polymerase chain reaction (RT-PCR) kit was obtained from TaKaRa (Tokyo, Japan).

Cell transfection

MCF-7 cells in the logarithmic growth phase were inoculated onto 6-well plate. When the cells reached a confluence of 50-60%, they were rinsed using serum-free DMEM/F12 medium. Serum-free DMEM/F12 medium was used for cell culture followed by the transfection experiment in the next day. Five μL of miR-200c mimics (100 pmol/L) and 2 μL of lipofectamine 2000 were resolved in 250 μL of serum-free DMEM/F12 medium, mixed by slight shaking and placed at room temperature for 5 min. Then, the above two solutions were mixed well and placed at room temperature for 20 min to form stable DNA-liposome complexes which were later added into the 0.5 mL serum-free DMEM/F12 medium. Four-six hrs later, DMEM/F12 medium containing 10% FBS was replaced.

Cell proliferation experiment

Cell counting kit-8 (CCK-8) experiment was used for detecting cell proliferation. MCF-7 cells in the logarithmic growth phase were inoculated onto 96-well plate for overnight culture. Cells were transfected using the method of lipofectamine 2000 and were divided into two groups, i.e. the control group and miR-200b overexpress-

ing group. At 6 hrs after transfection, CCK-8 reagent (10 μL/well) (Dojindo, Kumamoto, Japan) was added in each well for 2-hr incubation, followed by optical density $(OD)_{450}$ value detection using microplate reader. Three replicates were set for calculating the average of OD_{450} value and the detection was consecutively performed in 7 days. The cell growth curve was drawn to evaluate the viability of MCF-7 cells in different treatment groups.

Scratch wound healing assay

MCF-7 cells in the logarithmic growth phase were inoculated onto the 6-well plate in an appropriate cell density. When cell confluence reached 90%, cells were divided into two control groups and miR-200b mimics transfection group. After 6 hrs of culture, the culture medium was replaced. Then, scratch wounds were made by the tip of a pipette moving along the ruler to ensure that the lines were perpendicular. Subsequently, cells were washed for 3 times using phosphate buffered saline (PBS) to remove the unattached cells. Serum-free DMEM/F12 medium was replaced for cell culture. After treatment, cells were placed into an incubator for culture and photographed at 0, 12 and 24 hrs.

In vitro experiment of cell invasion using Transwell assay

MCF-7 cells in the logarithmic growth phase were inoculated into 6-well plate in an appropriate cell density. When confluence reached 90%, the cells were divided into control group and miR-200b mimics transfection group for 48 hrs of cell transfection. Fifty μL of matrigel (1 mg/mL) were coated onto the surface of each well in the upper chamber of Transwell and then the plate was placed into the incubator for agglomeration. We resuspended the transfected cells with serum-free culture medium, and cell density were adjusted into 1×10^5/mL. A hundred μL of suspension and 800 μL of DMEM/F12 containing 10% FBS were added into the upper and the lower chamber, respectively. Cells were then placed into the incubator for 24-hr culture, and methanol was used to fix the cells in the chamber at room temperature for 20 min. After fixation, chambers were gently rinsed using PBS, and the matrigel in the chamber and cells in the upper chamber were wiped using a cotton bud. Chambers were stained using 0.1% crystal violet at room temperature for 20 min. Thereafter, crystal violet was eluted using PBS, and the Transwell chambers were observed under microscope. Ten visions were selected for cell counting.

Western blot

MCF-7 cells were seeded into 6-well plate, in which phenylmethylsulfonyl fluoride (PMSF), inhibitor of phosphotransferase was added, and the radioimmunoprecipitation assay (RIPA) lysis (Beyotime, Shanghai, China) was performed. Equal volumes of proteins were added into the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred onto the polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after electrophoresis. The membrane was blocked for 1 hr using 5% skimmed milk at room temperature, followed by incubation using primary antibody overnight at 4°C. In the next day, we incubated the bands with HPS-labeled secondary antibody for 1 hr at room temperature. Thereafter, HRP-ECL was employed in the solution that was well mixed by illuminant A and B in 1:1 in the dark. The membrane, after being washed, was dried using filter paper to remove the eluent. The mixed illuminants were added onto the membrane which was then processed by Bio-Rad ChemiDoc MP Imaging System (Hercules, CA, USA) for luminescence and the stripes were analyzed by software.

RT-PCR

One mL of RNAiso Plus was added into the well-processed cells which were later blown and then placed on ice for 5 min. Then, cells were blown again, and transferred to a 1.5 mL Eppendorf tube, in which 200 μL of chloroform were added. Then the tube was vibrated for 30 s and the layered solution was then centrifuged at 12000 g/min for 10 min at 4°C. Thereafter, the supernatant was transferred into a new Eppendorf tube for mixing with 0.5 mL of pre-cooled isopropanol. After centrifugation at 12000 g/min for 10 min at 4°C, 0.5 mL of 70% ethanol that was prepared by the anhydrous ethanol and DEPC water were added into the solution. The precipitate was air-dried followed by centrifugation at 4°C, 12000 g/min for 5 min. RNA was resolved using an appropriate volume of diethyl pyrocarbonate (DEPC) water and the concentration and purification of RNA were assayed using micro-nuclei acid quantitative apparatus. Thereafter, reverse transcription was carried out following the protocols of the reverse transcription kit, and samples were added and amplified according to the instructions of the amplification kit.

Statistics

All experiment results were statistically analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All experimental results were expressed by mean ± standard deviation. The *t*-test was performed in the intergroup comparison and each result was analyzed at least 3 times. $P<0.05$ suggested that the difference was statistically significant.

Results

Upregulated expression of miR-200c in MCF-7 cells downregulated FUT4 expression

After MCF-7 cells were transfected with miR-200c mimics for 48 hrs, mRNA and protein levels of miR-200c and FUT4 were detected using qRT-PCR and Western blot. QRT-PCR results revealed that the miR-200c expression was elevated by miR-200c mimics transfection (Figure 1A), whereas FUT4 expression decreased ($p<0.05$, Figure 1B). Western blot results also showed decreased FUT4 expression after MCF-7 cells transfection with miR-200c mimics ($p<0.05$, Figure 1C). These results suggested that the upregulated miR-200c in breast cancer MCF-7 cells downregulates FUT4 expression.

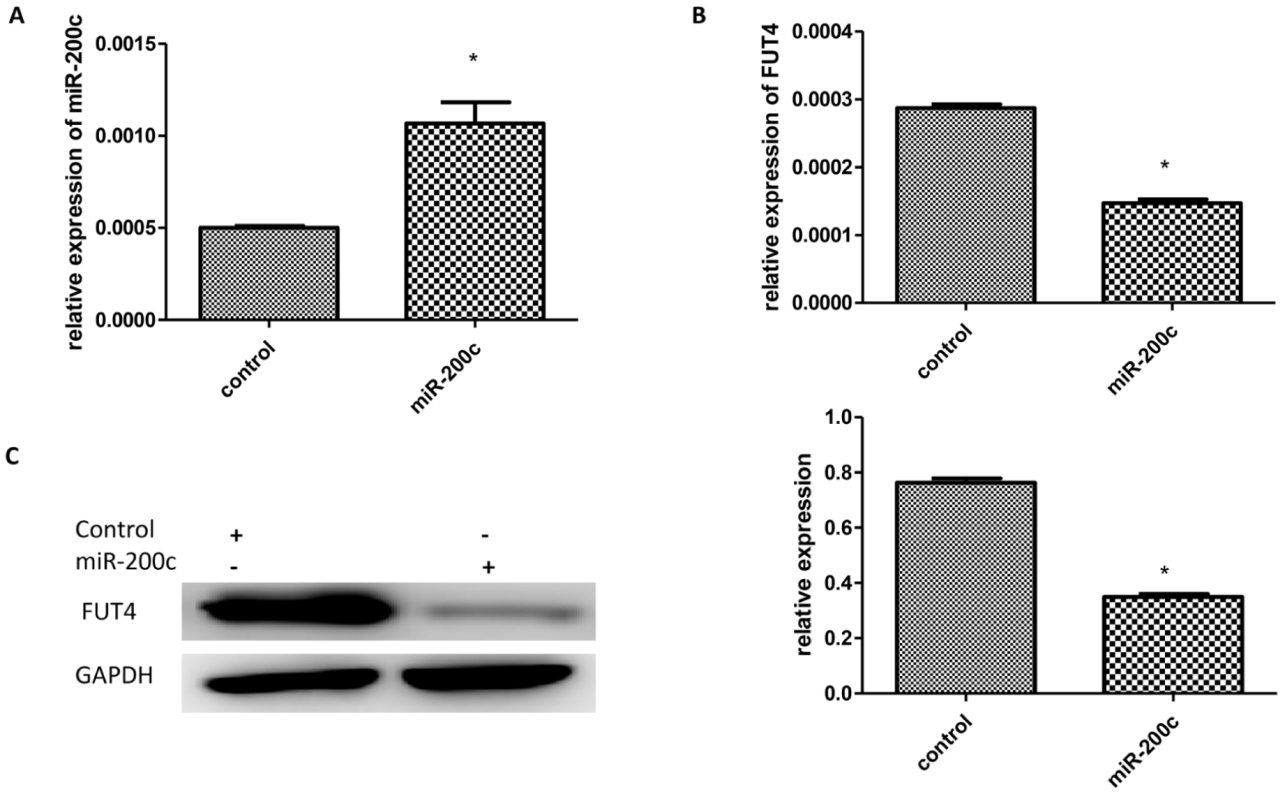

Figure 1. MiR-200c regulated FUT4 expression. **A:** Increased expression of miR-200c was detected after transfecting miR-200c mimics in MCF-7 cell by real-time PCR (*p<0.05). **B:** After miR-200c mimics transfection, decreased mRNA level of FUT4 was detected by real-time PCR (*p<0.05). **C:** The protein expression of FUT4 decreased after transfecting miR-200c mimics detected by Western blot (*p<0.05).

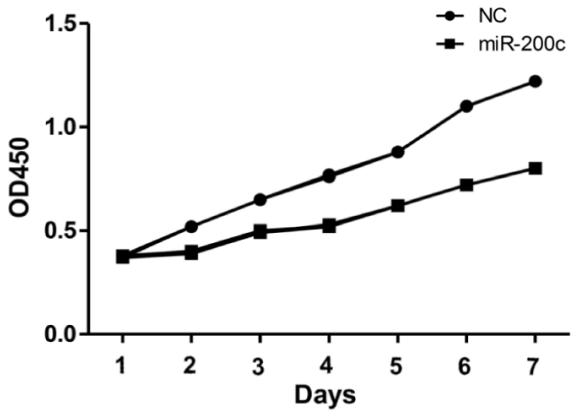

Figure 2. MiR-200c inhibited the proliferation ability of MCF-7 cells. Upregulation of miR-200c in MCF-7 cell decreased cell proliferation when compared with the control group (NC; p<0.05).

The effect of upregulated miR-200c expression on proliferation of MCF-7 cells

To detect the effect of miR-200c on the proliferation capability of MCF-7 cells, we carried out the CCK-8 cell proliferation experiment. After MCF-7 cells were transfected with miR-200c mimics, the growth rate became significantly slower than that of the control group (Figure 2). The experimental results showed that miR-200c could inhibit the proliferation of MCF-7 cells.

The effect of upregulated expression of miR-200c on migration of MCF-7 cells

We performed the scratch wound healing experiment to assess the effect of miR-200c on the cell migration capacity. We recorded the cell migrations at 0 and 24 hrs after the scratch wound after miR-200c mimics transfection and found the migration capacity of cells in the miR-200c mimics transfection group was lower than that in the control group (p<0.05, Figure 3). The results indicated that miR-200c could inhibit the migration of MCF-7 cells.

The effect of upregulated miR-200c expression on cell invasion

Transwell assay was carried out to detect the effect of miR-200c on the cell invasion capacity. The results were observed after 24 hrs of miR-200c mimics transfection and we observed that the invasion capacity of cells in the miR-200c mimics transfection group was significantly weaker than

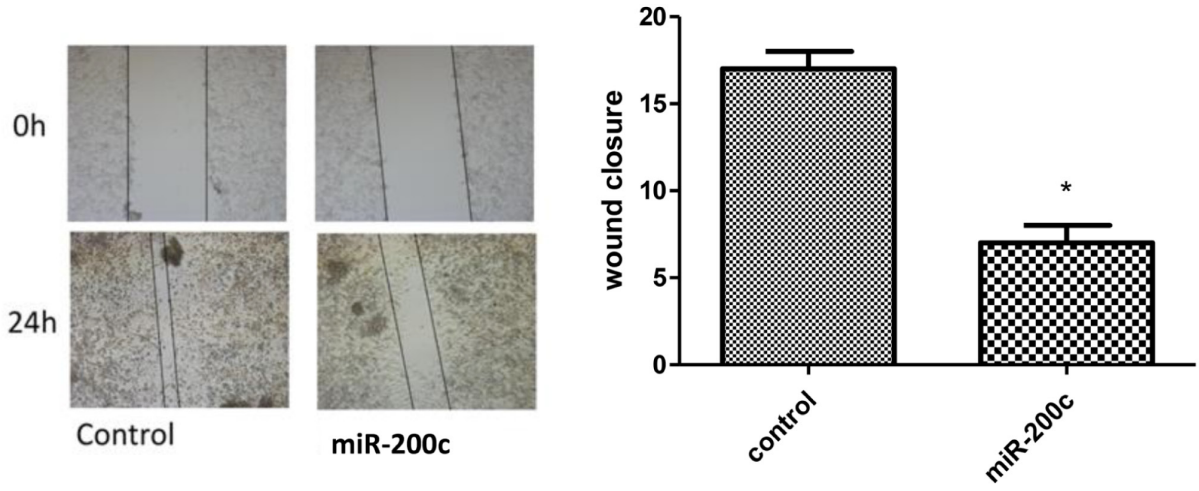

Figure 3. MiR-200c inhibited the migration ability of MCF-7 cells. The ability of migration declined with increased expression level of miR-200c in MCF-7 cells as observed by wound scratch assay (*p<0.05).

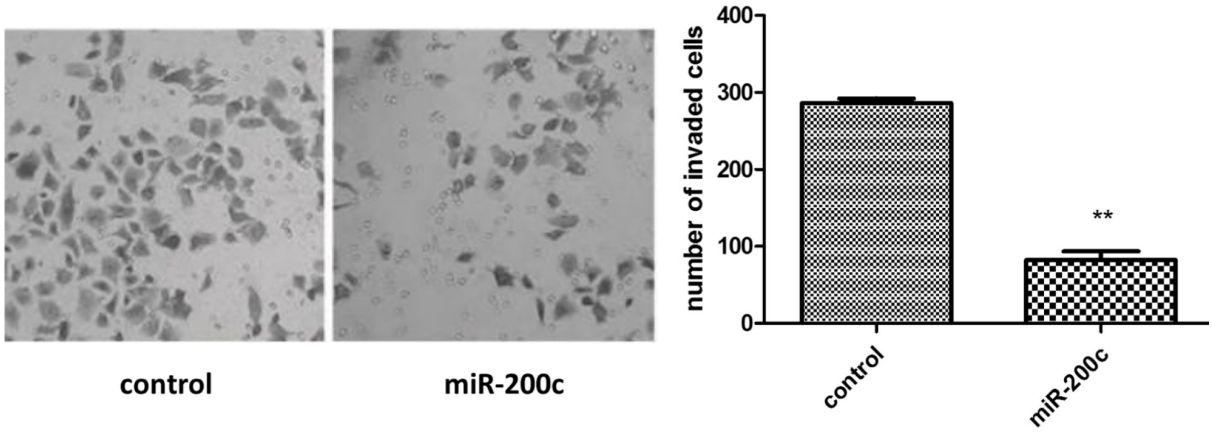

Figure 4. MiR-200c inhibited the invasion ability of MCF-7 cells. The ability of invasion declined with increased miR-200c expression as detected by invasion assay (Transwell) (**p<0.01).

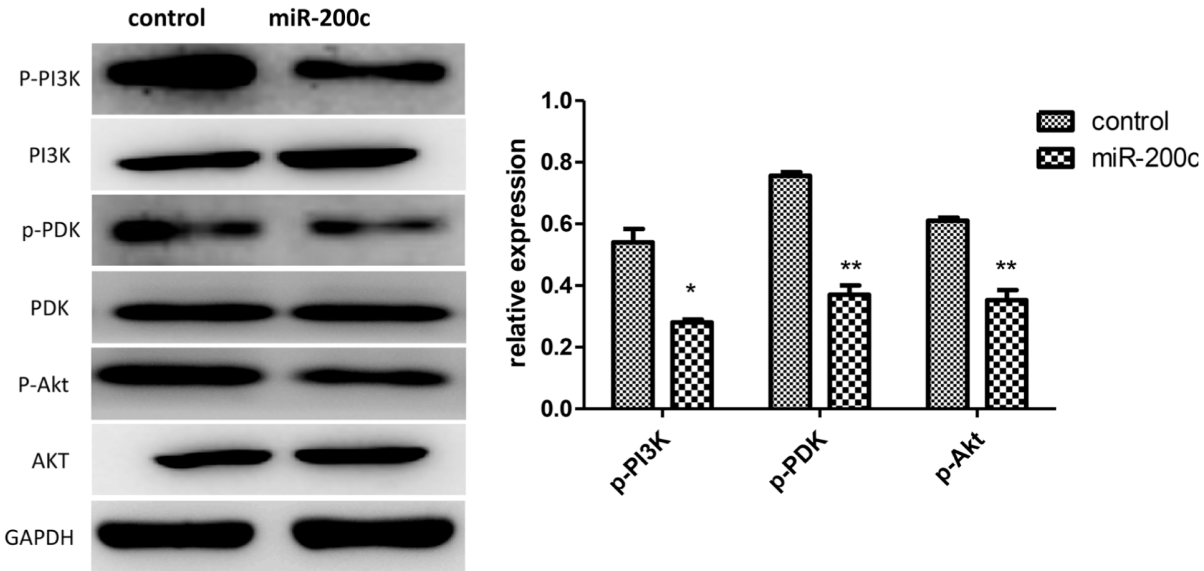

Figure 5. MiR-200c affected cell proliferation, migration and invasion through PI3K/AKT signaling pathway by targeting FUT4. Protein expressions of relative genes in PI3K/AKT signaling pathway decreased at phosphorylation levels in MCF-7 cells after miR-200c mimics transfection (*p<0.05, **p<0.01).

that in the control group ($p<0.05$; Figure 4). These results showed that miR-200c could inhibit the invasion ability of MCF-7 cells.

MiR-200c could act on FUT4 and affect cell proliferation, migration and invasion possibly through PI3K/AKT signaling pathway

Research has found that FUT4 could affect the functions of carcinoma cells, such as proliferation and invasion. PI3K/AKT signaling pathway might be involved in these biological processes of tumor cells. We speculated whether miR-200c could regulate the biological functions of MCF-7 cells through PI3K/AKT signaling pathway. To investigate this mechanism, we detected the protein expressions of FUT4 and key genes in PI3K/AKT signaling pathway, including p-PI3K, p-PDK and p-AKT. Figure 5 revealed that miR-200c overexpression downregulates the phosphorylation levels of PI3K, PDK and AKT, indicating the activated PI3K/AKT signaling pathway.

Discussion

The epidemiological studies on breast cancer have indicated that the median age at the onset of breast cancer in females in China is 48 years, showing a younger trend. Current treatments of breast cancer include surgery, chemotherapy, radiotherapy and endocrine therapy as well as targeted therapy, but there remains a number of limitations in efficacy. Thus, studies have been carried out on investigating the occurrence and progression of breast cancer, so as to search for diagnostic markers and new targets for effective management of this disease.

FUT4, mainly expressed in the epithelial cells and leukocytes [6], is a key enzyme which catalyzes the biosynthesis of LeY oligosaccharide antigens in the tumors that originate from the epithelial tissues [11]. Abnormal expressions of FUT4 have been identified in a variety of tumors. FUT4 is found to be highly expressed in gastric cancer [12], melanoma [13] and acute leukemia [14]. It is reported that PI3K/AKT and ERK/MAPK signaling pathways are closely associated with the lymph node metastasis of breast cancer [15]. Studies have also shown that upregulated expression of FUT4 could facilitate the proliferation and apoptosis [16] of tumor cells and regulate the cycle of tumor cells through MAPK and PI3K/AKT signaling pathways.

MiRs, a kind of small RNAs with key functions, exert regulatory functions in processes such as cell proliferation [17], cell apoptosis [18], tumor development and metastasis [19,20]. In the literature, the specific role of miR-200c in breast cancer is rarely reported. Some relative studies found miR-200c is lowly expressed in breast cancer, acting as tumor suppressor. In addition, there are few studies reporting the effects of miR-200c and FUT4 on the occurrence and development of breast cancer, and we maintain that studies should be performed to figure out whether miR-200c and FUT4 can serve as new targets in the treatment of breast cancer. In this study, we investigated whether miR-200c could affect the cell proliferation, migration and invasion of breast cancer cells through targeting FUT4 via the downstream PI3K/AKT signaling pathway. Based on the results taken, we identified the correlation between miR-200c and FUT4 to provide new ideas for the management of breast cancer.

We upregulated miR-200c in MCF-7 cells and found that the mRNA and protein expressions of FUT4 decreased. CCK-8 experiment indicated that the upregulated expression of miR-200c decreases the cell proliferation capacity. The scratch wound healing assay revealed that upregulated miR-200c decreases the cell migration capacity. Transwell assay revealed that miR-200c significantly weakened the invasion properties of cells. For the downstream of signal pathway, we found that expressions of relevant molecules in PI3K/AKT signaling pathway were altered upon the downregulation of FUT4, and the expressions of phosphorylated PI3K, PDK and AKT were also reduced after miR-200c mimics transfection. All these imply that miR-200c has a targeting effect on FUT4, and may participate in the cell proliferation, migration and invasion through PI3K/AKT signaling pathway. Based on the results of the above experiments, miR-200c and FUT4 could serve as new clinical indexes for evaluation of diagnosis and treatment of breast cancer.

Conclusion

In conclusion, miR-200c has a certain effect on the proliferation, migration and invasion in breast cancer. MiR-200c affects the biological functions of breast cancer cells by targeting FUT4 through the PI3K/AKT signaling pathway.

Conflict of interests

The authors declare no conflict of interests.

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