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MiR-20a suppresses proliferation and facilitates apoptosis of breast cancer cells *via* the MTOR signaling pathway

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Abstract. – OBJECTIVE: The paper aimed to explore the role of micro ribonucleic acid (miR)-20a in regulating the proliferation and apoptosis of breast cancer cells.

MATERIALS AND METHODS: The expression of miR-20a in breast cancer cells was analyzed *via* quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. Cell Counting Kit-8 (CCK-8) assay, colony formation assay, and flow cytometry were employed to analyze the proliferation and apoptosis of cells. Thereafter, the target proteins of miR-20a were predicted using TargetScan, a website for miRNA target gene prediction, and the interaction between miR-20a and the target genes was detected through the Luciferase reporter gene assay, qRT-PCR assay, and Western blotting. Finally, the miR-20a inhibitor and target gene expression plasmids were co-transfected for rescue experiment to study whether the target genes participate in the inhibitory effect of miR-20a on the proliferation of breast cancer cells.

RESULTS: It was found that the expression of miR-20a was upregulated in breast cancer cell lines. Silencing miR-20a expression inhibited the proliferation and promoted the apoptosis of breast cancer cell. Besides, it was demonstrated that late endosomal/lysosomal adaptor, mitogen-activated protein kinase (MAPK), and mammalian target of rapamycin (mTOR) activator 3 (LAMTOR3) were a direct target of miR-20a. The knockdown of LAMTOR3 expression repressed the influence of miR-20a on the proliferation of breast cancer cells.

CONCLUSIONS: MiR-20a targets LAMTOR3 gene to regulate the mTOR signaling pathway, thereby suppressing the proliferation and facilitating the apoptosis of breast cancer cells. It suggests that miR-20a exerts a carcinogenic effect in breast cancer, which may be a potential target for the treatment of breast cancer.

Key Words:
Breast cancer, MiR-20a, LAMTOR3, Proliferation, Apoptosis.

Introduction

Breast cancer is the second most common cancer around the world. According to global cancer statistics, there were more than 1.7 million new cases of breast cancer in 2012, accounting for 25% of the cases of all cancers[1]. Breast cancer can be induced by many factors, including genetic factors and lifestyle[2]. Besides, a study has shown that mutations of certain genes like breast cancer susceptibility gene 1 (BRCA1) and BRCA2 have a close correlation with the development of breast cancer, and mutations of BRCA1 and

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BRCA2 genes are often detected in breast cancer patients[3]. In addition, it is found that such genes as CHE2K, PALB2, and PIK3CA play a vital role in the progression of breast cancer[4]. Breast cancer-related genes and their mechanisms have been studied so far but studying the detailed regulation mechanism of breast cancer genes remains necessary due to the complexity and regulation process of tumorigenesis.

Micro ribonucleic acids (miRNAs) are highly conserved and modulate approximately 30% of human genes[5]. They were initially discovered in *Caenorhabditis elegans*, and then, detected in more and more species. Some studies[6-8] have manifested that miRNAs participate in various biological processes, including cell proliferation and differentiation, apoptosis, development of organisms, and pathogenesis of cancers. It is reported[9] that miR-15a/miR-16-1 cluster serves as a tumor suppressor in chronic lymphocytic leukemia, so such a cluster is usually deleted on chromosomes. Zhang et al[10] studied the expression and significance of miR-21 in gastric cancer tissues, as well as the role of miR-21 in biological behavior and gene of phosphate and tension homology deleted on chromosome ten (PTEN) expression in gastric cancer cells. The results manifested that miR-21 binds to PTEN and promotes the proliferation and invasion of tumors in gastric cancer. The levels of many miRNAs are dysregulated in breast cancer, including downregulated levels of miR-210, miR-19b, and miR-93 as well as upregulated levels of miR-10b, miR-145, and miR-195, leading to many dysfunctions[11-13]. Given this, miRNAs may be novel therapeutic molecular targets for the treatment of breast cancer.

As a member of the miR-17-92 gene cluster, miR-20a is proven in research to play a role in many types of cancer, which promotes the proliferation, migration, and invasion and inhibits the apoptosis of tumor cells, acting as an oncogene. Tang et al[14] studied whether the dysregulation of miRNAs is related to the pathogenesis of multiple myeloma and discovered through gene chip that miR-20a is highly expressed in the serum of patients with multiple myeloma, facilitates the proliferation and migration, represses the apoptosis and alters the cycle of multiple myeloma cells. In this study, the biological function of miR-20a in breast cancer was mainly explored to reveal new insights into the development and progression of breast cancer, providing a target choice for effective therapeutic strategies.

Materials and Methods

Materials

The main materials used were: Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS. St. Louis, MO, USA), miRNA extraction and isolation kit, miRNA complementary deoxyribonucleic acid (cDNA) first-strand synthesis kit and miRcute enhanced miRNA fluorescence quantitative detection kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China), miR-20a mimic, miR-negative control (NC), miR-20a inhibitor and anti_miR-NC (RiboBio, Guangdong, China), Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), cell counting kit-8 (CCK-8) assay kit (Dojindo Chemical Technology Co., Ltd., Shanghai, China), pmirGLO empty plasmid and dual luciferase detection system (Promega, Madison, WI, USA), anti-late endosomal/lysosomal adaptor, mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) activator 3 (LAMTOR3) and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Real Time-Polymerase Chain Reaction (PCR) instrument (Bio-Rad, Hercules, CA, USA), and apoptosis assay kit and FACScan flow cytometer (BD, Franklin Lakes, NJ, USA).

Cell Culture and miRNA Transfection

Human normal mammary epithelial cell line MCF-10A and human breast cancer cell lines (MCF-7, ZR-75-30, and MB-231) were cultured in DMEM containing 10% FBS and 1% double antibodies in a humidified 5% CO_2 incubator at 37°C. The cells growing well were taken for miRNA transfection using Lipofectamine 2000 according to the protocol of the manufacturer. The cells transfected for 48 h were used for subsequent assays.

MiRNA Extraction and Real Time-PCR

MiRNAs were extracted from cells using the miRNA extraction and isolation kit. A total of 500 pg of miRNAs were taken and reversely transcribed into first strand cDNAs following the instructions of the miRNA cDNA first-strand synthesis kit. Subsequently, cDNAs (0.5 μL) were taken as template, and quantitative Real Time-PCR (qPCR) assay for miR-20a was performed using the miRcute enhanced miRNA fluorescence quantitative detection kit, with U6 snRNA used as an internal control. The relative expression levels were calculated using $2^{-\Delta\Delta Ct}$. Finally, all samples

and genes were tested in triplicate. The primers used in this assay are listed as follows: miR-20a F: 5'-GCGGCGGTAAAGTGCTTATAGTG-3', R: 5'-TGCAGGGTCCGAGGTAT-3', and U6 F: 5'-CTCGCTTCGGCAGCACATATACT-3', R: 5'-ACGCTTCACGAATTTGCGTGT-3'.

Detection of Cell Proliferation Via CCK-8 Assay

Cell proliferation ability was detected using the CCK-8 kit. Firstly, the transfected cells were seeded into a 96-well plate with medium at 1×10^3 cells/well and cultured for 24, 48, 72, and 96 h in the humidified incubator at 37°C. Then, 10 μL of CCK-8 solution was added to the wells for 3 h of culture at different time points. A microplate reader was utilized to measure the spectral absorbance at 490 nm.

Colony Formation Assay

The obtained single cell suspension was inoculated into a 6-well plate at 300 cells/well. The medium was replaced every 4 d. After 12 d, the cell colonies were formed. After that, the colonies were fixed with 4% paraformaldehyde for 20 min, stained with 0.2% crystal violet, photographed, and counted.

Apoptosis Determined Through Flow Cytometry

The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit was used to determine the apoptosis rate. Next, 2×10^3 cells were harvested, suspended in 100 μL of binding buffer, and stained with Annexin V-FITC and PI for 10 min. Thereafter, 400 μL of binding buffer was added to the cell suspension, and the apoptotic cells were detected by flow cytometry.

Western Blotting

At 48 h after transfection, the cells were collected and washed twice with ice-cold PBS. Then, they were lysed with radioimmunoprecipitation assay (RIPA) cell lysis buffer (Beyotime, Shanghai, China) at 4°C for 30 min and centrifuged at 12000 × g for 10 min. After that, the supernatant was collected, and the protein concentration was determined by the BCA method. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 40 μg of total proteins in each lane. Next, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The PVDF membrane was blocked with 5% skim milk at room temperature for 1 h and incubated with primary antibody at 4°C overnight. Thereafter, the membrane was washed, added with secondary antibody and incubated in the dark at room temperature for 1 h. After that, the membrane was completely washed. Finally, the protein bands were visualized using enhanced chemiluminescent (ECL) reagents.

Detection of Luciferase Activity

LAMTOR3 3'-UTR fragments or mutant sequences containing miR-20a binding sites were synthesized and inserted into the pmirGLO vectors. The two plasmids were named pmirGLO-LAMTOR3-WT (wild type) and pmirGLO-LAMTOR3-Mut (mutant type), respectively, and co-transfected into cells with miR-20a mimic or miR-NC for 48 h. Next, the cells were collected, and the Dual-Luciferase reporter assay system was employed to determine the activity of firefly Luciferase and Renilla Luciferase. Renilla Luciferase activity was used as a standardized control.

Statistical Analysis

All data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and statistically analyzed using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). $p<0.05$ suggested statistically significant differences.

Results

Expression Level of miR-20a in Breast Cancer Cell Lines

Firstly, qRT-PCR assay was conducted to measure the relative expression level of miR-20a in three human breast cancer cell lines (MCF-7, ZR-75-30, and MB-231), with the expression level of miR-20a in human normal mammary epithelial cell line MCF-10A as the benchmark. The results (Figure 1) showed that the expression level of miR-20a was higher in the three human breast cancer cell lines than that in normal mammary epithelial cell line MCF-10A ($p<0.05$). It was hypothesized that miR-20a might act as an oncogene.

Effect of miR-20a Silence on Proliferation of Breast Cancer ZR-75-30 Cells

Next, the biological function of miR-20a was explored from the proliferative phenotype. The expression of miR-20a was inhibited by the trans-

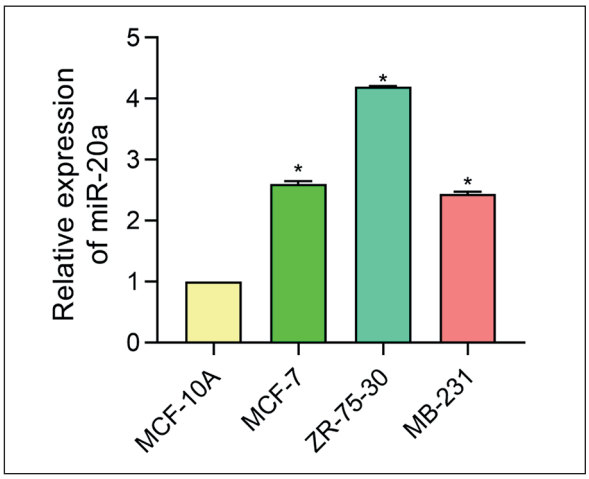

Figure 1. Relative expression level of miR-20a in MCF-7, ZR-75-30 and MB-231 to that in MCF-10A detected *via* real-time fluorescence qRT-PCR assay (*p<0.05).

fection with miR-20a inhibitor, and the effect of miR-20a silence on the proliferation of breast cancer ZR-75-30 cells was examined. The silencing efficiency of miR-20a was first examined by qRT-PCR. The results revealed that the efficiency of the designed and synthesized miR-20a inhibitor was significantly high (p<0.05) (Figure 2A). Based on CCK-8 assay, silencing miR-20a notably suppressed the proliferation of breast cancer ZR-75-30 cells (Figure 2B, p<0.05).

Effect of miR-20a Silence on Colony Formation in Breast Cancer ZR-75-30 Cells

Colony formation assay was carried out to further verify the effect of miR-20a silence on colony formation in breast cancer ZR-75-30 cells. It was found that compared with that in control group, the colony formation ability was remarkably weakened after silencing miR-20a (p<0.05) (Figure 3).

Influence of miR-20a Silence on Apoptosis of Breast Cancer ZR-75-30 Cells

The influence of miR-20a silence on the apoptosis of breast cancer ZR-75-30 cells was determined by flow cytometry. The results (Figure 4) showed that silencing miR-20a clearly increased the proportion of apoptotic cells (p<0.05), indicating that silencing miR-20a promotes the apoptosis of breast cancer cells.

MiR-20a Directly Targeted the 3′-UTR of the LAMTOR3 Gene

The potential target genes of miR-20a were predicted using the TargetScan database, and it was found that LAMTOR3 was one of the potential target genes (Figure 5A). LAMTOR3 is the mTOR activator 3, so it was investigated in this study. The LAMTOR3 3'-UTR binding site wild-type and mutant plasmids were constructed, and whether miR-20a can directly bind to the 3'-UTR of LAMTOR3 was observed through Dual-Luciferase reporter assay. As shown in Figure 5B, miR-20a mimic overtly inhibited the activity of wild-type Luciferase (*p<0.05) but had no significant effect on the activity of mutant Luciferase. Whether silencing or overexpressing miR-20a has an effect on the mRNA or protein level of LAMTOR3 was further detected *via* qRT-PCR and Western blotting. The results shown in Figure 5C and D revealed that the overexpression of miR-20a evi-

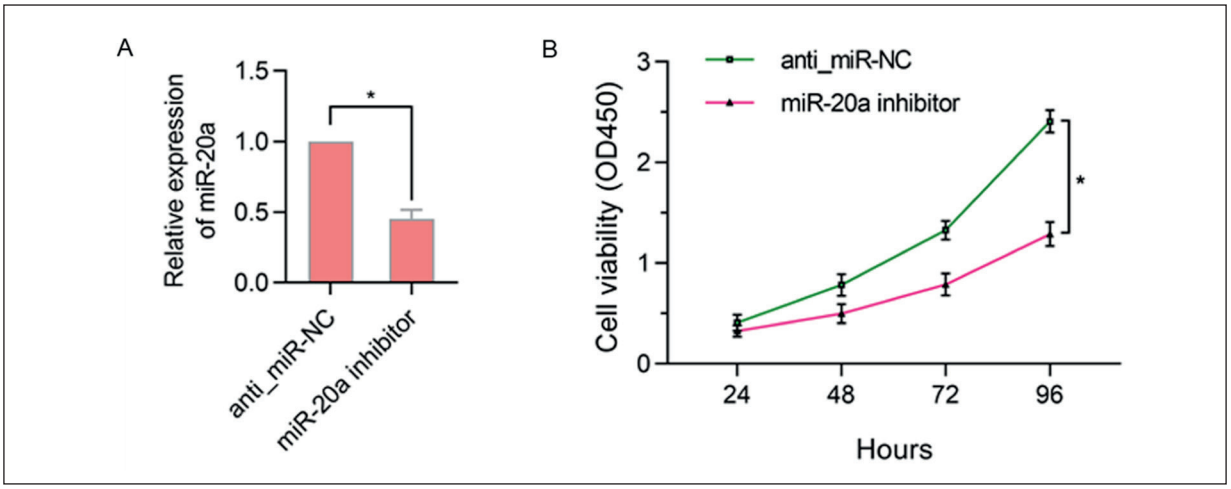

Figure 2. A, Silencing efficiency of miR-20a examined by real-time fluorescence qRT-PCR, **B,** Effect of miR-20a silence on the proliferation of breast cancer ZR-75-30 cells detected *via* CCK-8 assay (*p<0.05).

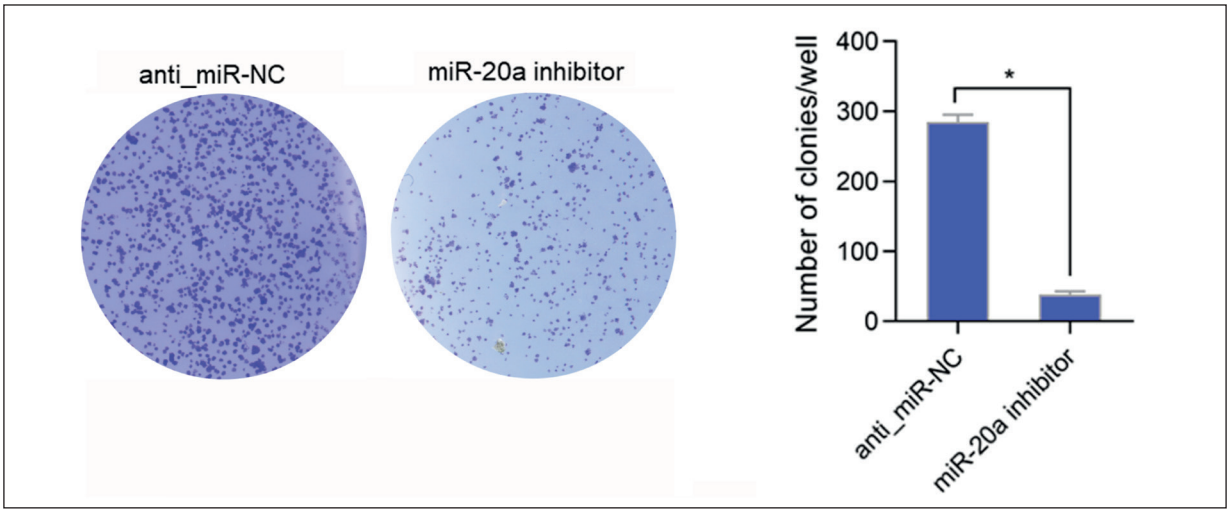

Figure 3. Effect of miR-20a silence on colony formation in breast cancer ZR-75-30 cells verified through colony formation assay (magnification: 10×) (*p<0.05).

dently repressed the mRNA and protein expression levels of LAMTOR3, while the silence of miR-20a exerted the opposite effect (*p<0.05). To sum up, these results suggest that miR-20a binds to the 3'-UTR of LAMTOR3 to suppress its expression.

Knockdown of LAMTOR3 Expression Blocked MiR-20a-Mediated Repression on Proliferation of Breast Cancer Cells

To further explore whether the inhibitory effect of miR-20a on the proliferation of breast cancer cells is mediated by LAMTOR3, miR-20a inhibitor and si-LAMTOR3 were co-transfected into the ZR-75-30 cells for rescue experiments. Then, CCK-8 assay was performed to detect cell proliferation activity. The results (Figure 6) showed that the knockdown of LAMTOR3 significantly weakened the inhibitory effect of miR-20a silence on cell proliferation (p<0.05), implying that miR-20a silence inhibits cell proliferation by targeting LAMTOR3.

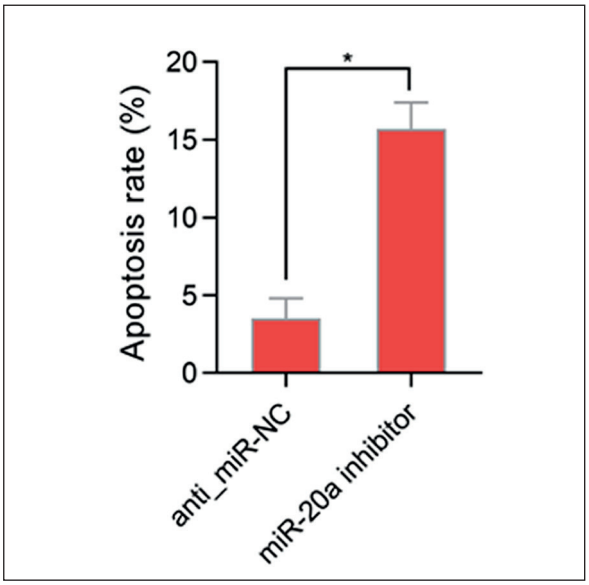

Figure 4. The influence of miR-20a silence on apoptosis of breast cancer ZR-75-30 cells determined by flow cytometry (*p<0.05).

Discussion

Breast cancer is the most common cancer in women, and many patients may have recurrence and metastasis. In China, its incidence rate is increasing at over twice the global growth rate, especially in urban areas[15]. As a subtype of breast cancer, triple-negative breast cancer lacks estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 (HER 2)[16]. Advance has been made in systemic chemotherapy, but the median survival of patients with metastatic breast cancer is still less than 2 years. Therefore, in addition to the existing surgical treatment, radiotherapy, chemotherapy, and immunotherapy in the treatment of breast cancer, researchers also need to find out more effective methods to early diagnose breast cancer, predict its prognosis, inhibit its metastasis, and reduce its mortality rate.

MiRNAs are non-coding single-stranded RNA molecules, which modulate target gene expressions through post-transcriptional processing[17].

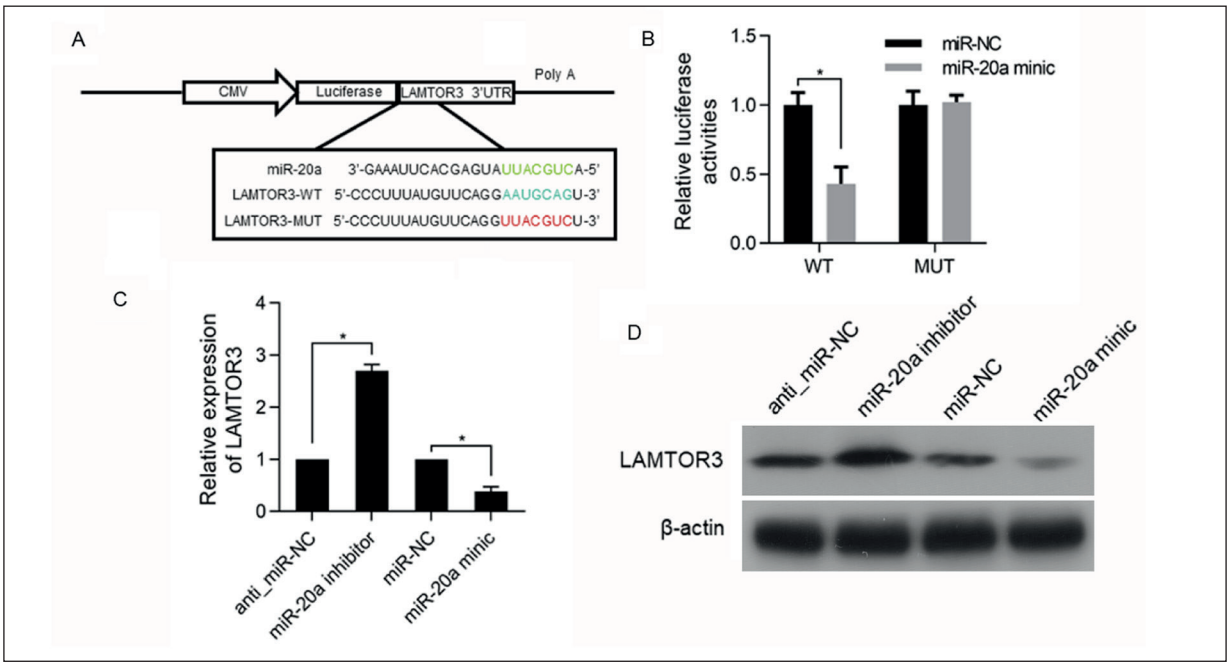

Figure 5. MiR-20a directly targets the 3'-UTR of the LAMTOR3 gene. **A,** Binding sites of miR-20a to the LAMTOR3 3'-UTR predicted using TargetScan website, **B,** Effect of miR-20a on LAMTOR3 luciferase activity determined *via* Dual-Luciferase reporter assay, **C,** and **D,** Changes in mRNA and protein expression levels of LAMTOR3 detected through qRT-PCR and Western blotting (*p<0.05).

The role of miRNAs under different physiological and pathological conditions is revealed in recent advances in cancer biology, and the aberrant expression of miRNAs is deemed to promote the progression of cancers by regulating key cancer-associated genes and signaling pathways. Zhang et al[18] investigated the role of miR-519b-3p in colorectal cancer and discovered that miR-519b-3p is distinctly downregulated in colorectal cancer clinical specimens and cell lines, and it regulates the uMtCK/Wnt signaling pathway to inhibit the proliferation and invasion of colorectal cancer. The role of miR-20a in a variety of tumors and its mechanism has been reported, however the research has not been reported in breast cancer. In this article we first found that miR-20a was significantly upregulated in breast cancer cells and attempted to explore the potential mechanism of miR-20a in the regulation of various biological functions in breast cancer.

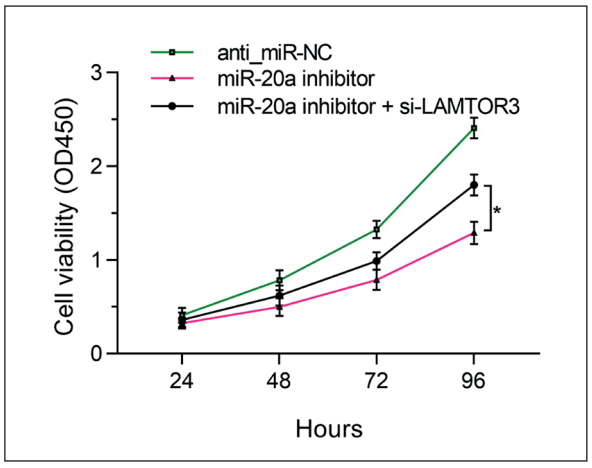

Figure 6. Proliferative viability of cells in different groups detected *via* CCK-8 assay (*p<0.05).

The purpose of this study was to explore the biological function and potential induction mechanism of miR-20a in the development and progression of breast cancer. It was found that the expression of miR-20a was upregulated in breast cancer cell lines, so it was assumed that miR-20a may serve as an oncogene in the development and progression of breast cancer. To verify such an assumption, phenotypic experiments, such as proliferation and apoptosis assays were conducted, and the results uncovered that silencing miR-20a significantly repressed the proliferation and promoted the apoptosis of breast cancer cells. It is believed that miR-20a plays a role in regulating the proliferation and

apoptosis of breast cancer cells and may play a good role in the diagnosis and prediction of breast cancer risk.

As a serine/threonine protein kinase, mTOR containing two functional complexes (mTORC1 and mTORC2) regulates intracellular signal transduction that is related to cell growth and survival as well as protein synthesis[19,20]. MTORC1 acts as a nutrient/energy/growth hormone sensitive modulator to exert active functions. The dysregulation of the mTOR signaling pathway is correlated with major morbid states, like diabetes mellitus, obesity, cancer, and cognitive deficits[19,20]. Malignant tumors are characterized by uncontrolled tumor growth, invasiveness, and metastasis, and the progression of malignant tumors involves several mutation events[21], including activation of oncogenes and inactivation of tumor suppressor genes. Besides, the mTOR signal usually mutates and thus is dysregulated in these cancer types by oncogenes and tumor suppressor factors[22]. Oneyama et al[23] demonstrates that mTOR may also be dysregulated due to changes in miRNA expression.

Conclusions

In this research, LAMTOR3, an mTOR activating factor, was detected as a target of miR-20a. It was directly shown *via* Luciferase assay that miR-20a bound to LAMTOR3 3'-UTR to inhibit expression, and the knockdown of LAMTOR3 expression blocked miR-20a-mediated inhibition on the proliferation of breast cancer cells, indicating that LAMTOR3 may play a crucial role in miR-20a-mediated inhibition on the proliferation of breast cancer cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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