Original Article

MicroRNA-3646 promotes cell proliferation, migration and invasion by targeting RhoA in breast cancer

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Abstract: MicroRNAs (miRNAs) are small single-stranded non-coding RNA molecules that are often located in genomic breakpoint regions and play an important role in regulating gene expression and other biological processes in human cancer, including tumor cell invasion and migration. MiR-3646 has been recorded to participate in tumorigenic progression in breast and bladder cancer, but in breast cancer its potential functions and exact mechanistic roles are still indistinct. In this study, we found that the expression level of miR-3646 in breast cancer cells and tissues was markedly increased when compared with normal breast cells and no tumor tissues. In addition, we found that miR-3646 promotes breast cells proliferation, migration and invasion. RhoA was identified as a direct target of miR-3646 by using luciferase reporter and western blot assays. The expression of RhoA and miR-3646 in the breast cancer cells had a positive correlation. Upregulation of miR-3646 in breast cancer cells increased RhoA protein level and downregulation of miR-3646 decreased the protein level of RhoA. Moreover we found that inhibit RhoA activity by C3 can block the miR-3646 to regulate RhoA.

Keywords: MiR-3646, breast cancer, proliferation, migration, invasion, RhoA

Introduction

Breast cancer is the most common cause of cancer related mortality among females with more than 508,000 deaths and it is the second most common cancer with nearly 1.67 million new cases diagnosed in 2012 [1, 2]. Breast cancer is a heterogeneous disease characterized by many kinds of pathological features [3]. Despite many great advances has made in the early diagnosis and treatment strategies of breast cancer, the long-term survival in patients, especially patients with metastatic breast cancer, remains ungratified [4]. The mortality of breast cancer increased derives from tumor metastasis [5]. It has been illustrated that tumor metastasis is a complicated, poly-stage and multi-factor process [6], and thus, it is very important to understand the molecular and cellular mechanisms that cause primary tumors to metastasize.

RhoA is a representative member of the Rho GTPase family, and plays a key role in the regulation of cytoskeleton reorganization. RhoA play an important role in many variety of biological process, such as cell motility [7], cytokinesis [8], morphogenesis and tumor development [9, 10]. Previous researchers have discovered that RhoA is over-expressed in several kinds of human cancers, including colorectal [11] and breast cancer [12], gastric cancer [13], and changing the expression level of intracellular RhoA can directly influence the process of metastasis and invasion in a variety of tumors [10, 14, 15].

MicroRNAs (miRNAs) are 20-25 nucleotides small non-coding RNAs that regulate the expression of hundreds of target genes through binding to the 3’-untranslated regions (3’-UTR) of their target mRNAs [16]. MiRNAs play important roles in many kinds of physiological and pathological processes, such as apoptosis, cell proliferation, differentiation, development, stress response and migration [17-19]. More and more evidence had demonstrated that miRNAs act as either tumor suppressors or promoters in the process of various tumors and play critical roles in regulating the
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posttranscriptional gene expression [20-22]. MiRNA-3646 has been found to be frequently upregulated and acted as a tumor promoter in many cellular functions [23]. The expression of miR-3646 in breast cancer and the relationship between the progression and development of it has not yet been elucidated.

In the present study, we aimed to detect the expression of miR-3646 in non-tumoral and tumoral breast tissue and cell lines and confirm the role of miR-3646 in breast cancer cell lines proliferation, colony formation and migration. In addition, we also want to identify RhoA as a target gene of miR-3646.

Materials and methods

Cell lines and patient samples

Human breast cancer cell lines MDA-MB-231, MCF-7 and a normal epithelial breast cell line MCF10A were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 and MCF-7 cells were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% FBS and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively). MCF10A cells were maintained in DMEM/F12 (1:1) medium supplemented with 5% horse serum, 100 ng/mL cholera enterotoxin, 0.5 mg/mL hydrocortisone, 10 µg/mL insulin and 20 ng/mL epidermal growth factor. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. The tissues were acquired from the cancer center of our hospital.

RNA extraction and real-time quantitative PCR

Total RNA was extracted by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the operating instructions. RNA was quantified using UV absorbencies at 260 and 280 nm (A260/280). Subsequently the RNA was reverse-transcribed into cDNA by using reverse transcription system (Thermo Scientific, CA, USA). The mRNA expression level of miR-3646 was detected by the ABI PRISM 7500 Sequence Detection System (ABI) using the TaqMan MicroRNA assay kits (Applied Biosystems, California, USA). U6 small nuclear RNA (snRNA) was used as the control normalize. The gene expression of RhoA also analyzed by SYBR Green and normalized with β-actin. The judgment of primer sequences’ specificity was based on dissociation curve, 2^ΔΔCt (cycle threshold) was used to calculate the relative gene expression levels.

Lentivirus production and infection

The miR-3646 NC (an oligonucleotide not on behalf of any known miRNA), miR-3646 mimic and miR-3646 inhibitor were purchased from Gene-Chem, Shanghai, China. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s protocols.

Plasmid construction and dual luciferase activity assay

The eukaryotic expression vector pcDNA3.1 (+) was subcloned with full-length RhoA cDNA which lacking the 3’-UTR (Invitrogen, Carlsbad, California, USA). The RhoA 3’UTR target site for miR-3646 was amplified by PCR and cloned into the Xhol site of pGL3 control (Promega, Madison, USA). This vector was called WT RhoA 3’UTR. The Quick-change mutagenesis kit (Strata-gene, Heidelberg, Germany) was used to carry out the site-directed mutagenesis of the miR-3646 target-site in the RhoA 3’UTR and known as Mut RhoA 3’UTR. For the luciferase activity assay, Wt or Mut RhoA 3’UTR vector and the control vector pRL-CMV (cytomegalovirus) coding for Renilla luciferase, Promega were cotransfected. Dual-Luciferase Reporter Assay System (Promega, Madison, USA) was used to detect the luciferase activity 36 hours after transfection.

Western blot and immunohistochemical analysis

Protein was extracted by using RIPA buffer which contain a protease inhibitor cocktail and phosphatase inhibitors (Sigma, St. Louis, MO, USA), according to the operating instructions. 50 µg of protein samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF, Millipore, Bedford, MA, USA) membranes using the Bio-Rad transfer system. Western blotting was performed using anti-β-actin and anti-RhoA (CST, Denver, CO, USA). The protein levels were detected with an ECL kit (Thermo Scientific, CA, USA) following the manufacturer’s instructions. Immunohisto-
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chemistry assays were conducted as previously mentioned [24].

Cell invasion assay

Cells transfected with the miR-3646 mimics, miR-3646 inhibitor and control oligo for 24 h. Then, the cells were plated onto the 24-well upper chamber with a membrane that was pre-treated with Matrigel (100 μg per well; BD Biosciences, San Jose, CA, USA). In the lower portion of the chamber, fresh medium contained 10% FBS was added. After the cells were cultured for 24 h at 37°C, we carefully

Figure 1. MiR-3646 is over-expressed in breast cancer cell lines and tissues. The expression level of miR-3646 in breast cancer cell lines and tissues was detected by using qRT-PCR. A. miR-3646 was markedly higher in breast cancer tissues (Tumor) when compared with adjacent non-tumor tissues (Normal), *P<0.05. B. miR-3646 was significantly increased in breast cancer cell lines (MCF-7 and MDA-MB-231) than that in the normal human breast cell line (MCF10A), *P<0.05, **P<0.01.

Figure 2. The expression level of miR-3646 in breast cancer cell lines transfected with miR-NC, miR-3646 mimic or miR-3646 inhibitor. A. The expression level of miR-3646 in MCF-7 cells, *P<0.01, **P<0.05. B. The expression level of miR-3646 in MDA-MB-231 cells, *P<0.01, **P<0.05.
removed the cells in the upper chamber. Invaded cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet, and counted under a microscope.

Cell proliferation assay

For the colony formation test, MDA-MB-231 and MCF-7 cells treated with miR-3646 NC, miR-3646 mimics, miR-3646 inhibitor were seeded in 6-well plates. Cultured after 14 days, the cells were stained with methylene blue to the colonies and then photographed.

Wound healing assay

Human breast cancer cells were seeded into 6-well plate. Wounds were created in the confluent cell monolayer using a 200 μl pipette tip, and any other free-floating cells and debris were removed by washing with PBS. Medium was then added, and the culture plates were incubated at 37°C. Wound healing within the scraped wound line was measured at a 48 h time point.

In vivo tumor growth assay

In this study all involved in animals procedures and experiments were performed in conformity to the National Institutes of Health Guide for Care and Use of Laboratory Animals. The 6-week-old nude mice [BALB/cA-nu (nu/nu)] were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed in the Animal Resource Facility at Laboratory Animal Centre. 5×10⁶ human breast cancer cells stably expressing either miR-3646 or miRNA control was injected subcutaneously in the both flanks of nude mice. Every two days to observe the palpable tumor formation. Thirty days after the implantation, removed the xenografts from mice and weighed the xenografts. Tumor volume was calculated using the following formula: 4π/3×(width/2)²×(length/2).

Statistical analysis

All data were presented as mean ± SD. Two-tailed student’s t-test was used to analyze significant differences between groups. P<0.05
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was accepted as significant. SPSS 18.0 and Graph Pad Prism 6.0 software were used for data analysis.

Results

MiR-3646 expression level is upregulated in both breast cancer cells and tissues

In order to declare the role of miR-3646 in tumor metastasis and tumor growth, we subsequently investigated the expression level of miR-3646 in breast cancer tissues and whether miR-3646 influenced the growth, invasion and migration of breast cancer cells. We first surveyed the level of miR-3646 in human breast cancer tissues. Results showed that the expression of miR-3646 in human breast cancer tissues was higher than that in normal breast tissues (Figure 1A). Furthermore, the expression level of miR-3646 in breast cancer cell lines MDA-MB-231 and MCF-7 was higher than in the normal epithelial breast cell

Figure 4. Wound healing assays indicates that miR-3646 promotes breast cancer cells migration, *P<0.01, **P<0.05 when compared with the miR-NC group.
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After we noted the high level expression of miR-3646 in breast cancer tissue and cells, we next examined the role of miR-3646 in the proliferation and invasion of breast cancer cells. MiR-NC, miR-3646 mimic, or miR-3646 inhibitor were transfected into MCF-7 and MDA-MB-231 cells and the expression of miR-3646 was detected by qRT-PCR. The expression level of miR-3646 in the mimic group was markedly increase when compared with the control, while in miR-3646 inhibitor group its expression remarkably suppressed in both cell lines (Figure 2A and 2B). The results illustrated that the level of miR-3646 can be regulated. In our colony formation assay, results showed that transfection of miR-3646 mimic obviously enhanced the proliferation of MCF-7 and MDA-MB-231 cells; on the other hand, inhibited the level of miR-3646 significantly suppressed the proliferation of MCF-7 and MDA-MB-231 cells (Figure 3A and 3B). Furthermore, the inva-

Figure 5. Expression of RhoA varies with the level of miR-3646 expression in breast cancer cells. A, B. Expression level of RhoA changes with the level of miR-3646 expression in MCF-7 cells, *P<0.01, **P<0.05 when compared with the miR-NC group. C, D. Expression level of RhoA changes with the level of miR-3646 expression in MDA-MB-231 cells, *P<0.01, **P<0.05 when compared with the miR-NC group.

Line MCF10A (Figure 1B). To sum up, the results showed that in human breast cancer tissues and cells the miR-3646 overexpressed.

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Expression of RhoA changes with the expression of miR-3646 in breast cancer cell lines

To verify that whether promote migration, proliferation and invasion by miR-3646 is related with changes in RhoA expression, we transfected the breast cancer cells with miR-3646 mimics or the inhibitor then detected the protein and gene levels of RhoA by western blot and RT-PCR. Results showed that, as the miR-3646 upregulated the levels of RhoA increased, when miR-3646 was downregulated the expression of RhoA decreased (Figure 5A-D). These results confirmed that miR-3646 regulated RhoA expression.

RhoA 3'UTR is a targeting gene of miR-3646

In order to study how miR-3646 regulated migration, we used miRBase databases continued to identify underlying targets known to play a part in cell mobility. Among the candidates studied, we found that the 3'UTR of the RhoA gene plays a critical role in cell stabilization as well as junction formation [25, 26], involves highly conserved parts that may act as a binding site for miR-3646 as determined at microrna.org (Figure 6A). To confirmed that RhoA is a direct target of miR-3646, RhoA wild-type (WT) or mutant 3'-UTR was subcloned into a luciferase reporter vector and co-transfected with miR-3646 mimics or negative control into breast cancer cells. The results demonstrated that in MCF-7 and MDA-MB-231 cell lines miR-3646 significantly inhibited the luciferase activity of the RhoA WT 3'-UTR but had no influence on the mutant (Figure 6B and 6C). These findings illustrated that RhoA is a direct downstream target for miR-3646 in breast cancer cells.

Inhibition of miR-3646 suppressed the growth of breast cancer cells in vivo

Based on the effect of miR-3646 promoting cell growth in both MCF-7 and MDA-MB-231 cells, we further measured the effect of miR-3646 tumor angiogenesis and growth in vivo. Transfection of MDA-MB-231 cells with miR-3646 inhibitor caused a significant decreased in the tumor volume and tumor weight of subcutaneous xenograft tumors on nude mice when compared with those cells who stably transfected with LV-control (Figure 7A-C). What is more, the expression level of RhoA was also declined by stable transfection of miR-3646 inhibitor (Figure 7D). In conclusion, these re-

Figure 6. RhoA 3’UTR is a potential miR-3646 target gene. A. Sequence alignment of miR-3646 and the RhoA 3'-UTR, which contains one predicted miR-3646-binding site. B. Luciferases say in MCF-7 cell lines co-transfected with miR-3646 a luciferase reporter containing the RhoA 3'-UTR (WT) or a mutant (Mut). C. Luciferases assay in MDA-MB-231 cell lines co-transfected with miR-3646 a luciferase reporter containing the RhoA 3'-UTR (WT) or a mutant (Mut).
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Results indicated that inhibition of miR-3646 could suppress angiogenesis and growth of breast cancer cells in vivo. After treated MDA-MB-231 cells with RhoA inhibitor C3 the migratory ability had no change with the levels of miR-3646 expression.

To further make sure that whether miR-3646 suppresses migration in a Rho-dependent manner, we using Rho inhibitor C3 at a final concentration of 2.0 μg/ml to blocked the activity of RhoA. Results showed that the RhoA mRNA and protein levels decreased after treatment with C3 when compared with the untreated cells (Figure 8A-C). Furthermore, after treatment with C3, the migratory ability of the MDA-MB-231 cells transfected with the miR-3646 mimics or the miR-3646 inhibitor was neither increased nor decreased when compared with the control cells (Figure 9). These results illustrated that miR-3646 does not regulate RhoA when the RhoA pathway is blocked.

Discussion

Breast cancer is the most common diagnosed cancer, which is caused by a lot of factors and is the leading cause of cancer-related death among women worldwide [27, 28]. In order to early diagnosis breast cancer and take appropriate treatment measures, we need to found a more exact method to discover the happening of breast cancer. With the development of molecular biology and biological detection technologies, molecular tumor markers of breast cancer have been given more and more attention, and associating clinical characteristics of individual with molecular markers of breast cancer for treatment has become possible [29-31]. Recently, there is a large number of evidence demonstrating that miRNA splay important roles in the tumorigenesis and metastasis [32, 33], and recognition of tumor-related miRNAs as well as their direct target genes is of great importance for understanding the biological meaning of miRNAs in breast cancer development and metastasis [34, 35]. Previous studies indicated that miR-3646 was over expressed in patients with bladder cancer, and breast cancer and it play a critical role in cell proliferation, invasion and metabolism [23, 36].

In this study, the results provided the evidence that miR-3646 accelerated cell proliferation and cell migration in breast cancer cell lines. First we researched the expression of miR-3646 in tumoral and non-tumoral breast tissues and cell lines. Results indicated that the expression level of miR-3646 was markedly increased in breast cancer cells and tumor tissues than that in normal breast cells and tissues. Up-regulation of miR-3646 significantly promoted cell proliferation, while knockdown of miR-3646 significantly suppressed cell growth. In order to further explore the effect of miR-3646 in breast cancer cells, we examined the role of miR-3646 in the growth of subcutaneous xenograft tumors in nude mice. In conformity to the in vitro researches, inhibit the expression of miR-3646 suppressed the growth of xenograft tumors. These results suggest...
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Rho/ROCK signaling channel has been verified to participate in the molecular mechanism of tumor metastasis [37]. Rho-subfamily protein is one of the members of the Ras superfamily with GTP enzyme activity, and mediates a wide variety of cellular effects, such as membrane trafficking, apoptosis/survival [38], cytoskeletal reorganization [39], proliferation, cell polarity, cell cycle, cell adhesion [40] and gene transcription. As a major member of the Rho GTPase family, RhoA has been found to be overexpressed in several types of human tumors, including breast [12, 41] and colorectal cancer [11, 42], hepatocellular carcinoma [43, 44] and gastric cancer [13]. Previous researchers have found that change the intracellular RhoA expression level can directly influence the process of invasion and metastasis in many cancers [10, 15]. The results of our study suggest that miR-3646 directly targets RhoA, and that the expression of RhoA change as the expression of miR-3646, and may be involved in the migration of breast cancer cells.

According to our results, we showed that in the breast cancer cell lines, miR-3646 directly modulated the target gene RhoA with the 3’UTR, finally inhibiting the RhoA expression, and thus controlling the migration and invasion of cells. In order to confirm that miR-3646 targeting of RhoA 3’UTR we used luciferase reporter and western blot demonstrated in breast cancer cells. In addition, we used Rho inhibitor C3 to block RhoA activity, results showed that miR-3646 was unable to regulate RhoA. These results further verified the possibility that miR-3646 inhibited migration via a RhoA-dependent pathway.
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Taken together, our results suggested that miR-3646 is involved in breast cancer cell lines and tissue specimens. In addition, we demonstrated that miR-3646 functions as an important role in the metastasis of breast cancer, at least, through upregulation of RhoA. This is the first study to combine miR-3646 with RhoA in breast cancers, and miR-3646 could be a potential target to treat breast cancers in the future.

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Disclosure of conflict of interest

None.

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