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MiR-99a suppress proliferation, migration and invasion through regulating insulin-like growth factor 1 receptor in breast cancer

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Abstract. – OBJECTIVE: Breast cancer is one of the most common malignant tumors in women. Despite the advances made in treatments of breast cancer, the incidence and death rates of breast cancer are still on the rise. Therefore, it is essential for us to explore the potential mechanism accounting for the malignancy of breast cancer.

MATERIALS AND METHODS: A lentiviral vector over-expressing miR-99a in MDA-MB-231 breast cancer cells was constructed. Cell proliferation was detected by MTT assay. Migration and invasion were measured by Scratch-wound assay and transwell assay. Direct target of miR-99a in MDA-MB-231 breast cancer cells was examined using bioinformatics and luciferase assay. The expression of endogenous insulin-like growth factor 1 receptor was quantified by qRT-PCR and Western blotting.

RESULTS: In our study, we found that miR-99a could suppress the proliferation, migration and invasion of MDA-MB-231 cell *in vitro* and inhibited the growth of xeno-transplant tumor *in vivo.* We also found that insulin-like growth factor 1 receptor (IGF-1R) was a direct target of miR-99a. Furthermore, knockdown of endogenous IGF-1R by siRNA could mimic the effect of miR-99a over-expression.

CONCLUSIONS: Our findings demonstrated that miR-99a could inhibit the malignancy of breast cancer cell by directly down-regulation of IGF-1R. These results indicated that miR-99a may be an important biomarker for prognosis and anticancer therapy in breast cancer in the future.

Key Words:
Breast cancer, miR-99a, IGF-1R, Carcinogenesis.

Introduction

Breast cancer, one of the most common malignant tumors, becoming the most threatened killer of women health in the world. About one hundred thousand women were diagnosed with breast cancer and half of them died in the United States in 2015, according to the latest statistics from American Cancer Society[1,2]. With the advances and progress made in chemotherapy, surgery, endocrine therapy, and molecular target therapy, the overall survival (OS) rates of breast cancer patients increased gradually compared to earlier years. However, breast cancer is still the second leading cause of cancer death in women[3]. Recurrence and metastasis are the main causes of highly cancer mortality in breast cancer patients, occurring in 20%-40% of patients treated according to NCCN guidelines each year[4]. Therefore, a better understanding of the regulatory mechanisms involved in these biological characteristics is urgently needed to overcome this devastating disease.

MicroRNAs (miRNAs) are small, endogenous non-coding RNAs composed of 19-23 nucleotides found in eukaryotic organisms, contributing to a variety of important physiological and pathological processes through post-transcriptional regulation of gene expression via mRNA degradation or expression inhibition of mRNA mainly by targeting the 3′-untranslated regions of mRNAs[5-7]. Numerous evidences reveled that miRNAs act as oncogenes or tumor suppressors in a variety types of cancer, such as lung cancer, prostate cancer, hepatic can-

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cer, and pancreatic cancer[8-11]. Moreover, a growing number of miRNAs were identified as critical regulators in human breast cancer. For example, by inhibiting translation of the messenger RNA encoding homeobox D10 (HOXD10), miR-10b can promote the invasion and metastasis of breast cancers[12]. Gwak et al[13] also indicated that miR-9 was highly expressed and associated with the epithelial-mesenchymal transition in breast cancer. More important, it has been reported that miR-99a was significantly reduced in breast cancer tissues and cell lines[14,15]. In addition, Wang et al[15] revealed that miR-99a could suppress the malignancy of breast cancer cells through regulating HOXA1. However, the underlying mechanism of mir-99a in breast cancer has not been fully identified so far.

The insulin-like growth factor 1 receptor (IGF-1R) is a trans-membrane protein belonging to the large class of tyrosine kinase receptors which is activated by insulin-like growth factor 1 (IGF-1) and IGF-2[17]. IGF-1R is ubiquitously expressed not only in normal tissues but also in many malignancies and plays a critical role in the development and progression in various human cancers[18,19]. In recent years, Law et al[20] indicated that activated IGF-1R may be expressed in all breast cancer subtypes, regardless of estrogen receptor (ER) or HER2 status. Furthermore, IGF-1R has been demonstrated to be the main downstream of many micro RNAs, such as miR-7, miR-145, miR-150, miR-181b, miR-223, miR-375, miR-630[17,21-23]. However, whether miR-99a has any function on IGF-1R remains unclear in breast cancer.

In this study, we revealed that over-expression of miR-99a could inhibit the proliferation, migration and invasion of breast cancer cell *in vitro* and suppress the xenograft growth *in vivo*. Meanwhile, we demonstrated that IGF-1R was a direct target of miR-99a in breast cancer.

Furthermore, down regulation of IGF-1R could mimic the function of miR-99a, suggesting that miR-99a is a potential tumor suppressor and maybe a promising therapeutic target for the treatment of breast cancer.

Materials and Methods

Cell Culture

Human breast cancer cell line MDA-MB-231 (Cell Bank of the Chinese Academy of Science, Shanghai, China) were maintained in Leibovitz's L15 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin, and 100 ng/mL streptomycin at 37°C in a humidified condition with 5% CO_2.

Lentivirus Packaging, Production and Establishment of Stable Cell Lines

A lentiviral packaging kit was purchased from Clontech (Mountain View, CA, USA). Pre-miR-99a synthesized from Genscript (Piscataway, NJ, USA) was cloned into the lentivirus vector pMSCV-puro. pMSCV-miR-99a or the control vector (empty vector), together with the package vectors psPAX2 and pMD2.G were co-transfected into HEK-293 cells. 72h later, the lentivirus-containing medium was collected and infected into MDA-MB-231 cells. For the selection of stable breast cancer cell lines, MDA-MB-231 cells were cultured in 1 μg/ml puromycin for 10 days after infection. Then the relative expression of miR-99a was confirmed by Quantitative Real Time PCR.

Knockdown of IGF-1R by small Interfering RNA (siRNA)

IGF-1R-specific small interfering RNA (siRNA-IGF-1R, 5'-CGACUAUCAGCAGCUGAAGTT-3') and its negative control siRNA (siRNA-NC, 5'-UUCUCCGAACGUGUCACGUdTdT-3') were designed and chemically synthesized from GenePharma Company (Shanghai, China). The siRNA duplex was transfected into MDA-MB-231 cells using RNA iMax (Invitrogen, Carlsbad, CA, USA) at a final concentration of 100 nmol/L in a serum-free medium according to the standard protocol.

RNA Extraction and Quantitative Real-time PCR

Total RNA from MDA-MB-231 cells was isolated using Trizol reagent (Invitrogen) and then reverse transcribed into cDNA by using AMV Reverse Transcriptase (Takara, Otsu, Shiga, Japan). Quantitative real-time PCR was performed using SYBR Green Master mix (Takara) with an Applied Biosystems 7300 Real Time PCR system (Foster City, CA, USA). Both RT and PCR primers were purchased from Ambion (Carlsbad, CA, USA). Beta-actin and U6 were used as internal control for sample loading and normalization. The 2-ΔΔCt method was applied to calculate the relative expression levels of miR-99a and IGF-1R.

Western-Blot

Total proteins of MDA-MB-231 cells were isolated using Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer's

instruction (Beyotime, Haimen, China). Protein samples were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Millipore Corp, Bedford, MA, USA). After blocking with nonfat milk, membranes were incubated with primary antibody against IGF-1R or GAPDH purchased from Santa Cruz Biotechnology (1:1000 dilutions; Santa Cruz, CA, USA) at 4°C overnight, followed by incubation with a secondary horseradish peroxidase-conjugated IgG (1:2000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting detection system (Millipore). Staining intensity of the bands was measured using a densitometer (Syngene, Braintree, UK) together with Genesnap and Genetools software (Syngene).

Luciferase Report Assay

The miR-NC or miR-99a expressed vector was co-transfected with IGF-1R-wild-type 3'UTR or IGF-1R-mutant-3'UTR plasmids(the seed region of miR-99a binding site was changed from UACGGGU to UACUUUA) in MDA-MB-231 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. 24 hours post-transfection, cells were harvested and the luciferase activity was measured by Promega (Madison, WI, USA) Dual-luciferase assay system. The values measured from the miR-99a expressed vector and wild-type 3'UTR plasmid were set as 100%.

Cell Proliferation Assays

3-(4,5)-dimethylthiahiazo(-z-yl)-3,5-di-phenytetrazoliumromide (MTT, Roche, Mannheim, Germany) was performed to measure the proliferation of breast cancer cells. All cells were seeded into 96-well plates (1×10^3 cells/well) and allowed to attach overnight. Each group contained six wells. On each day of five consecutive days, the medium was removed and 20 μl of MTT (5 mg/ml in PBS) was added to each well. After incubated at 37°C for 4 h, the supernatants were carefully aspirated, and 100 μl of dimethyl sulfoxide (DMSO) was added to stop the reaction. Absorbance values at 570 nm were measured on a Microplate Reader (Bio-Rad, Los Angeles, CA, USA).

Scratch-Wound Assay

Cells were seeded in 24-well plates and maintained until grown to confluence. Then, a wound was created manually using a 200 μl pipette tip. After washed with phosphate buffer saline three times, the floating debris was removed and the cells were further incubated for 48h. The migration distance was observed at two-time points (0 and 48 hours) using a reversed microscope (Olympus) at 100 × magnification. Image J was used to analysis the relative migration ability of cells (setting the gap width at 0 hour as 0%).

Invasion Assessment

The cell invasion ability was detected using a Transwell membrane (8μm pore size, Costar, Cambridge, MA, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). A total number of $1x10^5$ cells per well were cultured in the upper chamber, while 20% FBS was added to the lower compartment as a chemoattractant. Following 48 h incubation, non-invading cells were scraped by a cotton swab. The invaded cells were fixed with 100% methanol for 10 min, stained in 0.5% crystal violet for 20 min, and then photographed by a light microscope (Olympus, Tokyo, Japan) at 200 x magnification. Also invaded cells were then immerged in 33% ice-cold acetic acid for 10 min and absorbance at 570 nm was assessed by a microplate reader (Bio-Rad). The values of negative control were set as 100%.

Tumor Formation Assay

Two stable cell lines ($1x10^7$ cells) were suspended in 200 μl of PBS and injected subcutaneously into the left and right back of 4-week-old nude mice respectively (Cancer Institute of the Chinese Academy of Medical Science). Tumor volumes were measured once every 4 days for 28 days using the following formula: volume = length x width^2/2. Later, all mice were sacrificed and tumors were removed and weighed.

Statistical Analysis

Two groups were compared using Student's *t*-test, whereas analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used in order to compare more than two groups. All dates were processed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). $p<0.05$ was considered to indicate a statistically significant difference.

Results

Over-Expression of miR-99a Suppresses Proliferation, Migration and Invasion of MDA-MB-231 Cells

To further test the effects of miR-99a on breast cancer cells, MDA-MB-231 cells were infected

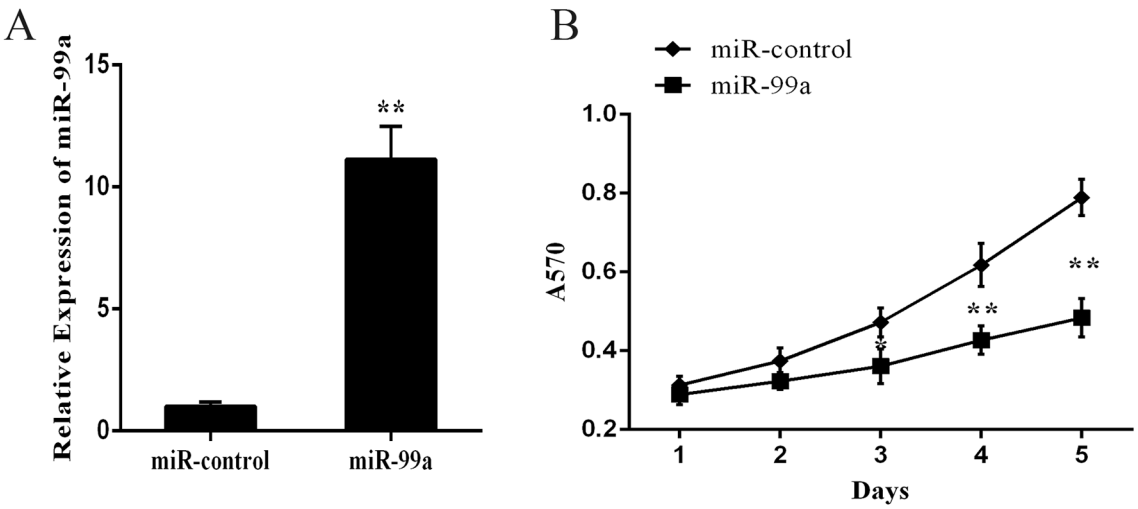

Figure 1. Up-regulation of miR-99a suppresses proliferation of MDA-MB-231 cells *in vitro*. ***(A)*** The relative expression of miR-99a was detected by qRT-PCR in MDA-MB-231 cells. The average miR-99a expression was normalized to U6 expression. ***(B)*** Cell proliferation rates were measured at different time points (1, 2, 3, 4 and 5 days) and the absorbance at 570 nm was recorded. Data are shown as mean ± SEM (n=3), *$p < 0.05$, **$p < 0.01$.

with a lentivirus expression pre-miR-99a. First, qRT-PCR was utilized to detect the expression levels of miR-99a. As shown in Figure 1A, miR-99a transcripts were increased markedly after lentivirus infection, compared to control cells ($p < 0.01$). Then, the function of miR-99a on the proliferation of breast cancer cells was detected by MTT assay. The results suggested that the proliferation slightly changed within 0-3 days. Within 3-5 days, miR-99a caused inhibition of cell proliferation significantly when compared with controls ($p < 0.05$, Figure 1B). Meanwhile, the scratch-wound assay was performed to evaluate cell migration capability. Compared with the control group, over-expression of miR-99a greatly decreased the scratch wounds closure and inhibited cell migration rate ($p < 0.01$, Figure 2A). Furthermore, cell invasive ability was assessed by matrigel-coated transwell chambers. The results in Figure 2B showed that the invasive capacity of MDA-MB-231 cells was reduced obviously after up-regulation of miR-99a ($p < 0.01$). Taken together, above results demonstrated that miR-99a could repress the proliferation, migration and invasion of breast cancer cells.

Over-Expression of miR-99a Inhibits Xenograft Growth in vivo

To examine whether miR-99a have any impact on the development and progression of breast cancer *in vivo*, we inoculated MDA-MB-231 cells expressing miR-NC or miR-99a into the left and right flank of nude mice and recorded the tumor volumes once every four days by a caliper till the end of this experiments, 28 days of post-induction. The results are consistent with the observation *in vitro* that miR-99a inhibits the proliferation of MDA-MB-231 cells ($p < 0.05$, Figure 3A). At the time of sacrifice, a significant difference of tumor weight was observed between miR-NC- and miR-99a-expressing breast cancer cells, tumor mass was suppressed greatly in miR-99a-expressing MDA-MB-231 cells ($p < 0.01$, Figure 3B).

miR-99a Directly Targets IGF-1R 3'UTR in Breast Cancer Cells

We used two algorithm programs PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/) to determine the potential direct targets of miR-99a. The results suggested that IGF-1R, which is considered as a key regulator in the pathogenesis and treatment of breast cancers[17-19], has a putative binding sites of miR-99a (Figure 4A) and may be a candidate target of miR-99a. Firstly, qRT-PCR and western blot analysis were applied to examine the effect of miR-99a on the mRNA and protein expression levels of IGF-1R. As revealed in Figure 4B and 4C, up-regulation of miR-99a markedly decreased the expression levels of IGF-1R in MDA-MB-231 cells. To further confirm our hypothesis, IGF-1R-wild-type 3'UTR and IGF-1R-mutant-3'UTR

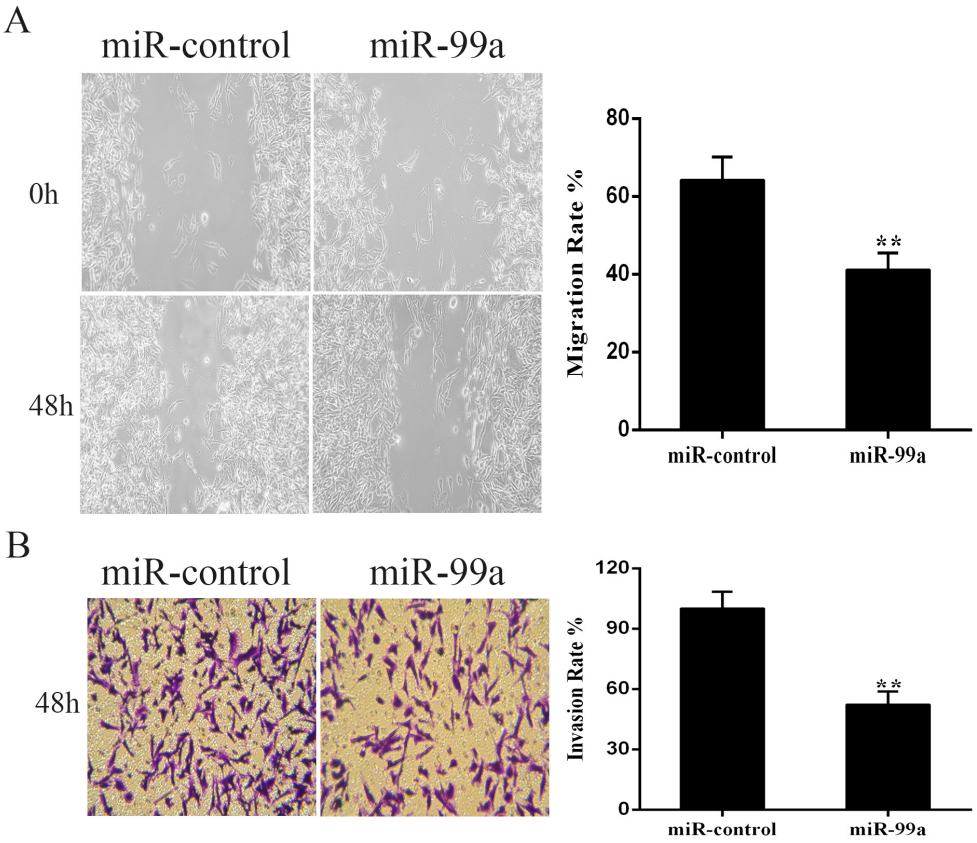

Figure 2. Over-expression of miR-99a inhibits migration and invasion in MDA-MB-231 cells. ***(A)*** Scratch-wound assay was applied to detected cell migration rate. Gap distances were measured at time point 0 and 48 h to quantify migration rate. ***(B)*** Invasion capability was measured by transwell assay in MDA-MB-231cells. Data are shown as mean ± SEM (n=3), $^{**}p < 0.01$.

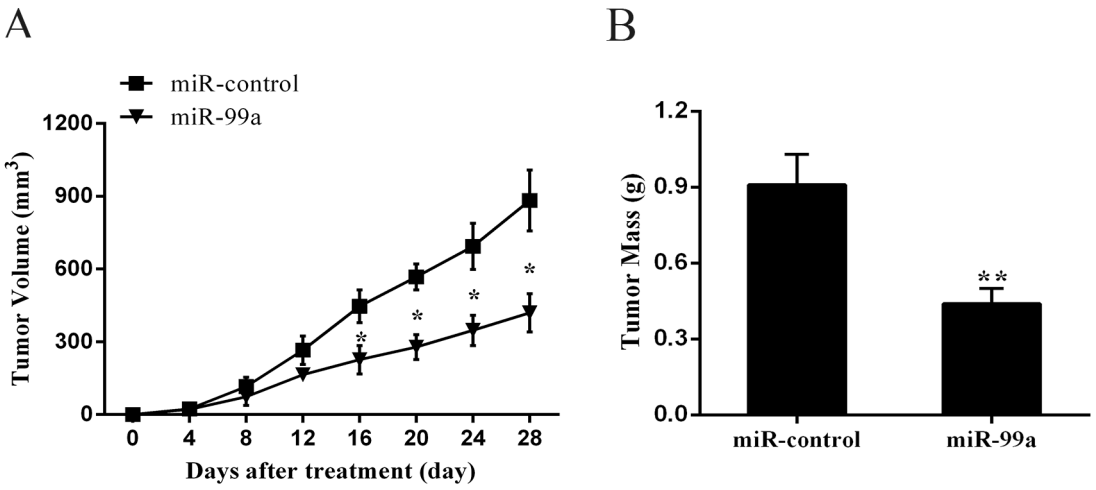

Figure 3. Over-expression of miR-99a repressed the growth of established tumors *in vivo*. ***(A)*** MDA-MB-231cells were injected subcutaneously into BAL B/c nude mice. The tumor volumes were noted lasting 4 weeks. ***(B)*** Tumor-bearing mice were sacrificed and the tumor weights were measured. Data are shown as mean ± SEM (n=3), $^{*}p < 0.05$, $^{**}p < 0.01$.

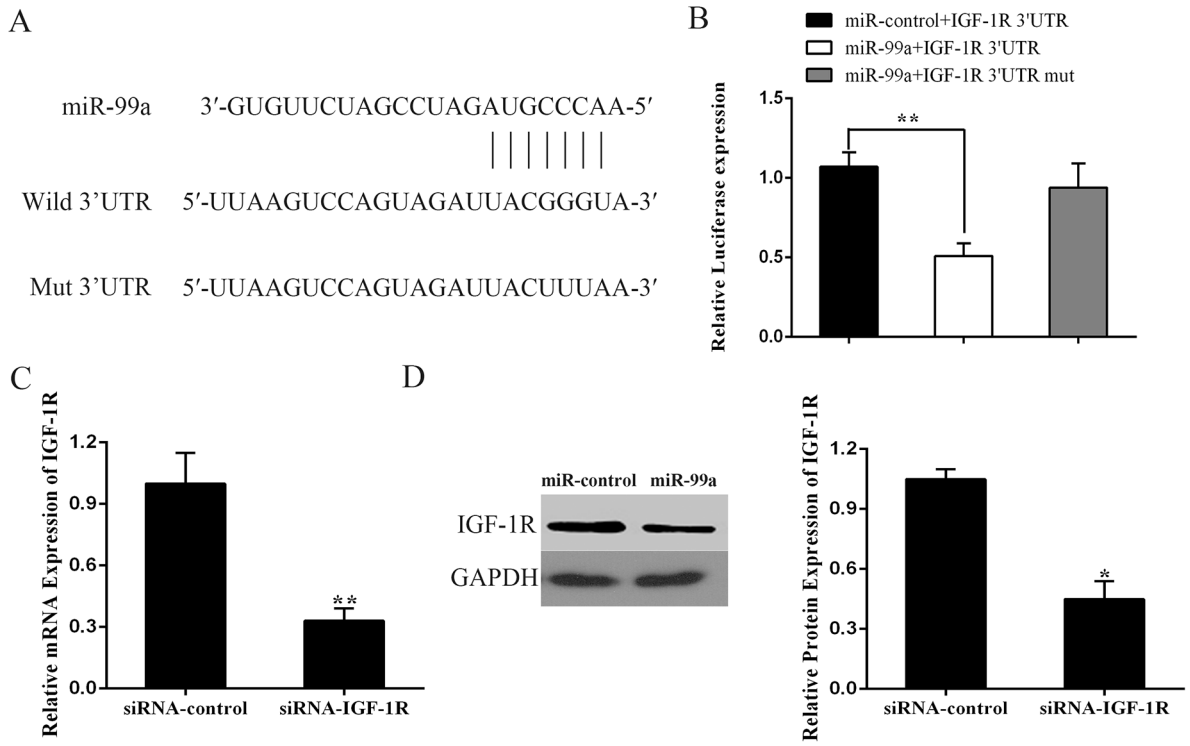

Figure 4. IGF-1R is identified as a direct target of miR-99a *in vitro*. ***(A)*** Sequences of miR-99a-binding site within 3'UTR of IGF-1R and mutated nucleotides in 3'UTR of IGF-1R. ***(B)*** The relative luciferase activity was detected by Dual Luciferase Reporter Assay System 48h after transfection. ***(C-D)*** qRT-PCR and western blot were performed to analyze the expression of IGF-1R in mRNA and protein levels in MDA-MB-231 cells. Data are shown as mean ± SEM (n=3), $^{*}p < 0.05$, $^{**}p < 0.01$.

luciferase reporter plasmid was constructed and co-transfected with miR-99a or miR-NC expression vector. As a consequence, the Luciferase activity was significantly lower when miR-99a was up-regulated by miR-99a expression vector, whereas the Luciferase expression level with the IGF-1R-mutant-3'UTR was not affected by miR-99a (Figure 4B). These results suggested that IGF-1R is a direct downstream of miR-99a in breast cancer cells.

IGF-1R Plays a Critical Role in miR-99a-Mediated Malignancy in Breast Cancer Cells

With the purpose of identifying the role of IGF-1R on the malignancy of breast cancer cells, RNAi approach was used to down-regulate IGF-1R expression. The data of qPCR and Western blot assay showed that the endogenous expression levels of IGF-1R were repressed significantly (Figure 5A and 5B) post-transfection of specific interfering IGF-1R in MDA-MB-231 cells. The effects of IGF-1R siRNA on the proliferation of breast cancer cells was detected by MTT assay. As shown in Figure 5C, compared to the control group, knockdown of IGF-1R resulted in a significantly decreased proliferation of MDA-MB-231 cells (Figure 5C). Also, we used Scratch-wound assay and Transwell assay to check the migratory ability and invasiveness of breast cancer cells. As shown in Figure 6A and 6B, down-regulation of IGF-1R impeded the migratory ability of MDA-MB-231 cells. Corresponding effects on invasiveness were also observed in the Transwell assay. These results are in keeping with the previous findings that over-expression of miR-99a can suppress malignancy of breast cancer cells. In conclusion, these data provided further evidence that IGF-1R is a direct and functional target of miR-99a in breast cancer cells.

Discussion

In recent years, multiple researches have strongly supported miRNAs are important players not only in the numerous important cellular functions, including proliferation, differentiation,

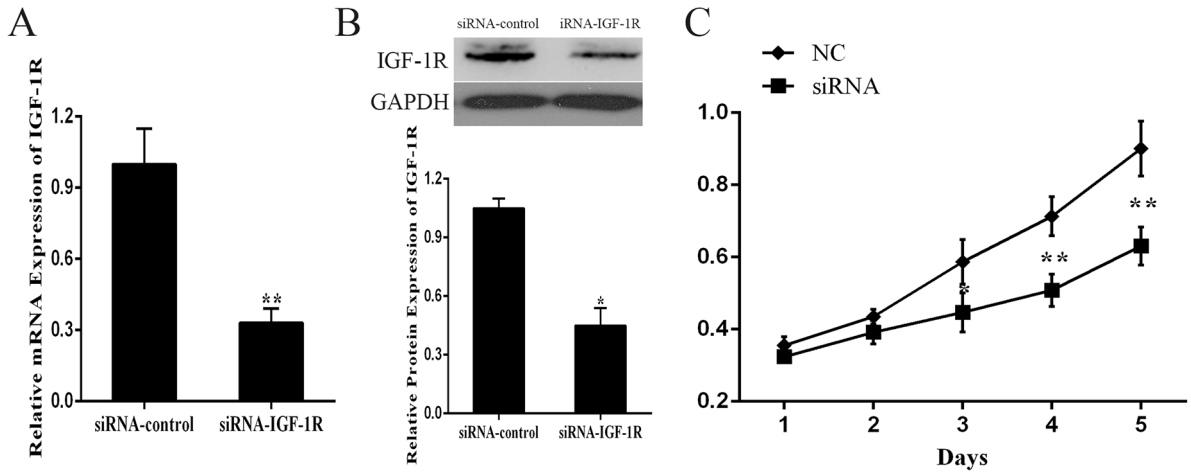

Figure 5. Down-regulation of IGF-1R suppresses cell proliferation of MDA-MB-231 cells. ***(A-B)*** The mRNA and protein expression level of IGF-1R were detected by qRT-PCR and Western blotting assay in MDA-MB-231 cells after treated with siRNA. ***(C)*** The cell proliferation of MDA-MB-231with different expression level of IGF-1R was analyzed by MTT assay. Data are shown as mean ± SEM (n=3), *$p < 0.05$, **$p < 0.01$.

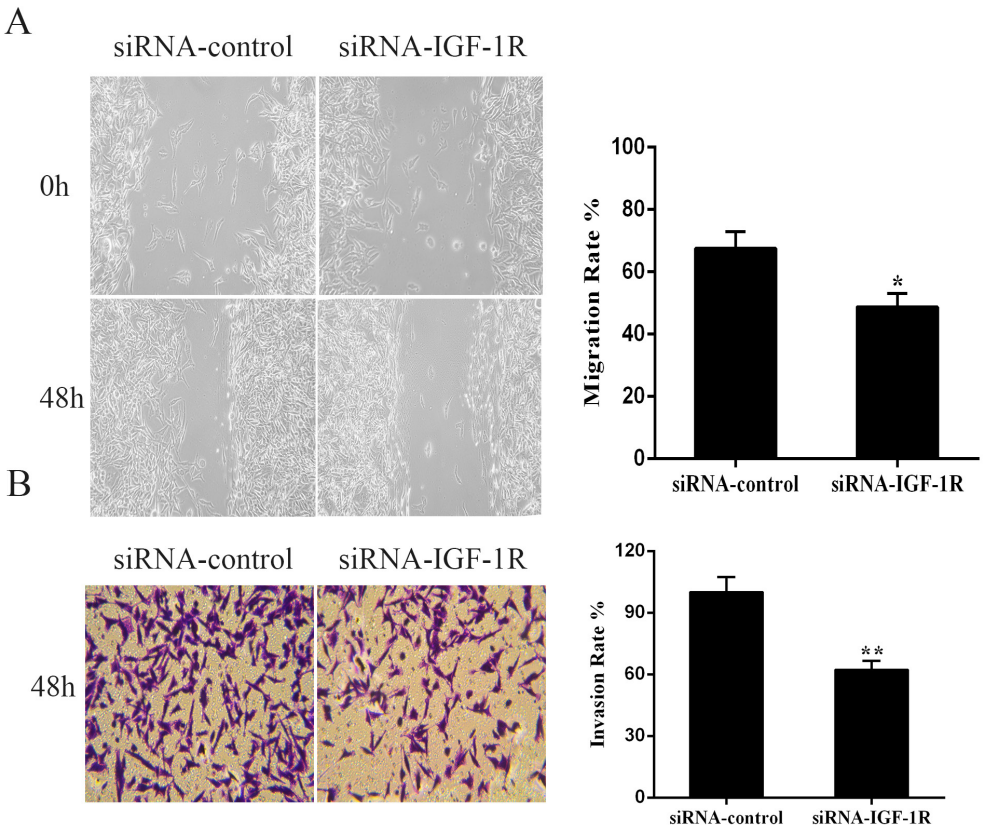

Figure 6. Down-regulation of IGF-1R suppresses MDA-MB-231 cell migration and invasion. ***(A)*** Scratch-wound assay in MDA-MB-231cells with different expression level of IGF-1R. Gap distances were measured and images were obtained at time point 0 and 48 h to quantify migration rate. ***(B)*** Transwell assay in MDA-MB-231cells with different expression level of IGF-1R was measured. Data are shown as mean ± SEM (n=3), *$p < 0.05$, **$p < 0.01$.

cell cycle control and apoptosis but also in the initiation and progression of tumors[24-27].

Abnormal expression of miRNAs may cause dysregulation of oncogenes or tumor suppressor genes, subsequently affecting the genesis and progression of variety types of cancer, such as lung cancer, breast cancer, prostate cancer, hepatic cancer, and pancreatic cancer[8-10,28,29]. Recently, miR-99a was reported as a key regulator in tumorigenesis, multiple studies[30-31] have demonstrated miR-99a act as tumor suppressor in many types of cancers, including NSLC, bladder cancer, prostate cancer, anaplastic thyroid cancer, oral cancer and cervical cancer. In breast cancers, miR-99a was acknowledged to be a tumor suppressor via targeting different targets[14,16]. However, the intracellular environments of different cells are diversity. Whether IGF-1R can be regulated by miR-99a in breast cancer needs to be explored, making a contribution to the new therapeutic target in breast cancer.

In recent years, growing evidence shows that IGF-1R and its ligands may contribute to human cancer progression[34]. Abnormal expression of IGF-1R could active various downstream signaling pathways, such as PI3K/AKT, MAPK/ERK, EP2/EP4, RAS/RAF/ERK signaling pathways[35-39], which are key mediators of the proliferation and apoptosis in malignant tumors. Moreover, aberrant expression of IGF-1R may involve in the progression of epithelial-mesenchymal transition (EMT) and function as an important regulator of cell migration and invasion[17].

In this study, we confirmed that over-expression of miR-99a could markedly repressed breast cancer cell proliferation, migration and invasion *in vitro* and decreased the growth of implant tumors *in vivo*. The mRNA and protein level of IGF-1R was down-regulated after up-regulating of miR-99a. Also, we applied luciferase reporter assay and the result showed that miR-99a could bind to the 3'-UTR of IGF-1R efficiently. Moreover, knockdown of IGF-1R could also suppress MDA-MB-231 cells' proliferation, migration and invasion. These results observably proved that miR-99a directly targets IGF-1R and functions as a tumor suppressor in breast cancer.

Conclusions

Our findings demonstrated that miR-99a directly down-regulating IGF-1R and plays a critical role in the proliferation, migration and invasion of breast cancer cell. These results not only indicate that miR-99a may serve as a tumor suppressor gene involved in breast cancer pathogenesis, but also provide a rationale for miR-99a regarded as an important biomarker for prognosis and anticancer therapy in breast cancer in the future.

Acknowledgement

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Conflicts of interest

The authors declare no conflicts of interest.

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