Original Article

MicroRNA-3941 targets IGF-1 to regulate cell proliferation and migration of breast cancer cells

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Abstract: We aimed to investigate the effects and regulatory mechanism of microRNA-3941 (miR-3941) in the progression of breast cancer. The expression of miR-3941 and insulin-like growth factor 1 (IGF-1) was determined in breast cancer tissues and cell lines. A miR-3941 mimics, inhibitor, and scramble RNA were individually transfected into the cancer cells. Then, the effects of overexpression and suppression of miR-3941 on cell viability, migration, and invasion, as well as on the expression of epithelial-mesenchymal transition (EMT)-related proteins (E-cadherin, N-cadherin, and vimentin) were further investigated. In addition, luciferase reporter analysis was performed to confirm whether IGF-1 was the potential target of miR-3941. Small interfering RNA targeting IGF-1 was transfected into the cells to further investigate whether miR-3941 regulated the breast cancer cell migration and invasion by targeting IGF-1. The inverse expression of miR-3941 (downregulated) and IGF-1 (upregulated) was observed in the breast cancer tissues and cells. The overexpression of miR-3941 significantly inhibited the breast cancer cell viability and suppressed cell migration and invasion. In addition, IGF-1 was confirmed as the target of miR-3941, and IGF-1 expression was negatively regulated by miR-3941. The knockdown of IGF-1 significantly reversed the inhibitory effects of miR-3941 overexpression on cell migration, invasion, and the EMT-related proteins. Our results indicate that miR-3941 is downregulated in breast cancer cells, and the downregulation of miR-3941 may promote breast cancer cell proliferation, migration, and invasion through not targeting IGF-1 expression. miR-3941 and IGF-1 may serve as diagnostic markers or potential targets for breast cancer treatment.

Keywords: Breast cancer, microRNA-3941, insulin-like growth factor 1, cell proliferation, cell migration, cell invasion

Introduction

Breast cancer is a phenotypically and genetically complex disease and the most frequently diagnosed cancer in women worldwide [1-3]. In 2012, the World Health Organization estimated that there were approximately 1.68 million cases of breast cancer and 522,000 related deaths [4]. Moreover, metastatic breast cancer is incurable by primary surgery [5]. Therefore, the elucidation of the key molecular mechanisms underlying breast cancer will facilitate the early diagnosis and development of a promising therapeutic approach.

MicroRNAs (miRNAs), endogenous small non-coding RNAs (approximately 21-23 nucleotides in length), can negatively regulate their target genes at the post-transcriptional level [6, 7]. miRNAs are key regulators frequently implicated in a wide range of biological or pathological processes, including tumorigenesis [8]. In breast cancer, various miRNAs such as miR-139-5p, miR-33b, miR-27b, miR-204, etc. [12-15] and their roles are receiving more and more attention [9-11]. The identification of key miRNAs associated with breast cancer progression has become a hot topic of research. Recently, miR-3941 has been identified to regulate the malignant progression of lung adenocarcinoma via targeting immunoglobulin-binding protein 1 (IGBP1) expression [16]. The interactions between ATP-binding cassette subfamily A member 6 (ABCA6) and miR-3941 have also been found to participate in tumorigenesis of colorectal cancer [17]. However, it is largely unknown whether miR-3941 plays a key role in the progression of breast cancer.
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In the present study, we investigated the expression of miR-3941 in breast cancer tissues and cells. Then, the effects of overexpression and suppression of miR-3941 were further investigated on cell viability, migration, and invasion. Besides, the regulatory relationship between miR-3941 and insulin-like growth factor 1 (IGF-1) was explored. Our findings elucidate the crucial role and possible regulatory mechanism of miR-3941 in the progression of breast cancer.

Materials and methods

Patients

From February 2014 to March 2016, a total of 37 patients were enrolled in this study, who were diagnosed with breast cancer and underwent surgery in our hospital. The diagnosis of breast cancer was confirmed according to the previous description by McDonald and his colleagues [18]. Cancer tissues and their matched normal tissues were collected, quickly frozen in liquid nitrogen, and stored at -80°C for further experiments. The procedures used in this study were approved by the Ethics Committee of the Shandong Provincial Hospital, and all patients provided their informed consent.

Cell culture and cell transfection

The human breast cancer cell line MDA-MB-231 and breast epithelial cell line MCF-10 (European Collection of Cell Cultures, Wiltshire, UK) were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) containing 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 10% fetal bovine serum (FBS) at 37°C in an incubator with 5% CO₂. Cell transfection was then performed. In brief, cells were seeded in a 6-well plate at a density of 5 × 10⁵ cells/well and cultured for 24 h before transfection. Cell transfection was then performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The miR-3941 mimics, miR-3941 inhibitor, scramble RNA, and IGF-1-specific small interfering RNA (siRNA) were synthesized by Sangon Biotech (Shanghai, China).

Cell proliferation assay

Cell viability was evaluated using an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] assay. In brief, 5 × 10³ cells per well were grown in a 96-well plate. At 24, 48, 72, and 96 h after transfection, 20 μL of MTT was added to each well, and the incubation of the plate continued for 4 h. The MTT/medium mixture was rapidly removed, and then 150 μL of dimethyl sulfoxide was added to each well to dissolve the formazan crystals formed in viable cells. The absorbance (570 nm) of cells in each well was measured using a spectrophotometer (Olympus, Japan). Each experiment was carried out independently three times.

Colony assay

Cell proliferation was also assessed using a clonogenic assay. In brief, the cells at a density of 100 cells/dish were grown in tissue culture dishes (60 mm) in RPMI-1640 medium supplemented with 10% FBS for 14 days. Afterward, the cells were fixed, stained with the Diff-Quick stain, and air-dried. The colonies that contained more than 30 cells were counted under a microscope (IX83, Olympus). Each experiment was carried out in triplicate.

Transwell assay

Cell migration and invasion were examined with a Transwell assay. The membranes of Transwell chambers (8-μm pore size; Corning, USA) used for the invasion assay were precoated with a diluted extracellular matrix solution (Sigma-Aldrich, Shanghai, China). In brief, after transfection, the cells at a density of 5 × 10⁴ cells/well were grown in the upper chamber with a serum-free medium. Then, the lower chamber was filled with a medium containing 10% FBS, which is considered a chemoattractant. After incubation for another 48 h at 37°C, non-migrated or non-invaded cells remaining in the upper chamber were scraped using cotton swabs, while migrated or invaded cells were fixed, stained with the Diff-Quick stain, and counted under a microscope (IX83, Olympus). Each assay was repeated three times.

Luciferase reporter assay

The target relationship between IGF-1 and miR-3941 was evaluated using a dual-luciferase reporter assay. The full-length IGF-1 3′-UTR containing the miR-3941 binding site was inserted into the XbaI-site of the pGL3 vector (Promega, Madison, WI, USA) to construct the pGL3-IGF-3′-UTR-WT reporter. The pGL3-IGF-
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![Figure 1. The inverse expression of miR-3941 and IGF1 in breast cancer tissues and cells. A: The expression of miR-3941 in breast cancer tissues and their adjacent normal tissues. B: The expression of miR-3941 in breast cancer MDA-MB-231 cells and normal breast MCF-10 cells. C: The expression of IGF1 in breast cancer tissues and their adjacent normal tissues. D: The expression of IGF1 in breast cancer MDA-MB-231 cells and normal breast MCF-10 cells. Error bars indicate means ± SD. *, P < 0.05 and **, P < 0.01.]

3'-UTR-MUT reporter, containing a mutated miR-3941 binding sequence, was synthesized using a site-directed mutagenesis kit (Stratagene, CA, USA). Afterward, cells (1 × 10⁶) were co-transfected with 50 pmol of the miR-3941 inhibitor (or control miRNA), 1 μg of pGL3-IGF-3'-UTR-WT or pGL3-IGF-3'-UTR-MUT, and a Renilla luciferase expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 36 h of transfection, luciferase activity was measured using the Dual-Luciferase® reporter assay system (Promega). Renilla luciferase activity served as an internal control.

Quantitative reverse transcription-polymerase chain reaction

The extraction of total RNA was performed with the Trizol reagent (Takara, Japan) in accordance with the standard protocol. The purity and concentration of the isolated RNA were measured using a SMA 400 UV-VIS spectrophotometer (Merinton, Shanghai, China). Then, the purified RNA (0.5 μg/μL) was reverse-transcribed into cDNA using the PrimeScript 1st strand cDNA synthesis kit (Invitrogen). The expression of target transcripts was measured in the tissues or cells using the SYBR ExScript qRT-PCR kit (Takara, Japan) in an Eppendorf Mastercycler.
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Melting curve analysis was set at the end of each polymerase chain reaction (PCR) to confirm that a single product was amplified. Each reaction was performed in triplicate, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression levels. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control.

Western blotting

Total protein extracts were obtained by lysing the cells in each group with radioimmunoprecipitation assay buffer (Sangon Biotech, China) supplemented with phenylmethanesulfonyl fluoride (Sigma, USA), and then protein concentrations were measured using a BCA protein assay kit (Pierce, Rochford, IL, USA). Afterward, 25 μg of protein was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore). After blocking the membranes for 1 h with Tris-buffered saline with Tween 20 (TBST), containing 5% non-fat milk, appropriate primary antibodies were added, and the membranes were incubated overnight at 4°C. Subsequently, the membranes were incubated for 1 h with a horseradish peroxidase-labeled goat anti-rat secondary antibody (1:1,000 dilution). After rinsing the membranes three times with TBST, protein bands were detected using an enhanced chemiluminescence kit (Millipore Corp., Bedford, MA, USA). GAPDH served as a loading control.

Statistical analysis

All data were expressed as the mean ± standard deviation, and statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Differences between two groups were evaluated using Student’s t-test. Differences among groups were calculated by one-way analysis of variance, followed by Turkey’s post-hoc test. A P-value of < 0.05 indicated statistical significance.

Results

Inverse expression of miR-3941 and IGF-1 in breast cancer tissues and cells

The expression of miR-3941 in breast cancer tissues was low compared to that in the adjacent normal breast tissues (P < 0.01; Figure 1A). In vitro cell analysis showed that miR-3941 was also expressed at a lower level in MDA-MB-231 cells in comparison with the expression in normal breast epithelial MCF-10 cells (P < 0.01; Figure 1B). In addition, IGF-1 was highly expressed in both breast cancer tissues and in MDA-MB-231 cells compared to the expression in the adjacent normal tissues and normal cells, respectively (P < 0.01; Figure 1C, 1D). These data revealed the inverse expression of miR-3941 and IGF-1 in breast cancer tissue and cells.

miR-3941 overexpression inhibited breast cancer cell viability

To further explore the relationship between the dysregulation of miR-3941 and breast cancer, MDA-MB-231 cells were transfected with the miR-3941 mimic and miR-3941 inhibitor. After transfection, we found that the expression of miR-3941 was significantly upregulated in the miR-3941 mimic group and markedly downregulated in the miR-3941 inhibitor group compared with the expression in the control or scramble group (P < 0.05; Figure 2A), indicating that miR-3941 was successfully overexpressed and suppressed, respectively, in breast cancer MDA-MB-231 cells. Moreover, the results of the MTT assay showed that miR-3941 overexpression resulted in a significant decrease in the cell viability compared with that in the control group or scramble group, while opposite effects were observed after miR-3941 suppression (P < 0.05; Figure 2B). Besides, the results of the colony assay were consistent with those of the MTT assay, showing that the number of colonies significantly decreased after the miR-3941 overexpression and obviously increased after the miR-3941 suppression (P < 0.05; Figure 2C and 2D). These data indicated that miR-3941 overexpression significantly inhibited the breast cancer cell proliferation.

miR-3941 overexpression significantly suppressed the migration and invasion of cancer cells, possibly via regulation of EMT-related proteins

The effects of dysregulation of miR-3941 on cell migration and invasion were evaluated using the Transwell assay. As shown in Figure 3A-D, in comparison with that in the scramble group or control group, the number of migrated
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Figure 2. The effects of miR-935 on cell proliferation. A: The expression of miR-3941 in different transfection groups. B: MTT assay showing the cell viability of different transfection groups. C and D: Colony assay showing the number of the colony of different transfection groups. Error bars indicate means ± SD. *, P < 0.05 and **, P < 0.01 (compared with control group).

or invaded cells significantly decreased after miR-3941 overexpression and markedly increased after miR-3941 suppression (P < 0.05). Moreover, in comparison with that in the scramble group or control group, the expression of E-cadherin was significantly upregulated after miR-3941 overexpression, while those of N-cadherin and vimentin were downregulated (P < 0.05; Figure 3E and 3F). Opposite trends in the expression of these proteins were observed after miR-3941 suppression (P < 0.05; Figure 3E and 3F). These data indicated that the miR-3941 overexpression might suppress cell migration and invasion via upregulation of E-cadherin and downregulation of N-cadherin and vimentin.

IGF-1 is the target of miR-3941

We further investigated whether IGF-1 was regulated by miR-3941. First, the prediction results obtained using the TargetScan (http://www.targetscan.org/) showed that IGF-1 was a potential target for miR-3941 (Figure 4A). We, therefore, conducted a luminescence reporter assay. The results showed that the relative luciferase activities of cells carrying IGF-1 3’-UTR-WT significantly decreased after miR-3941 overexpression compared to those of control cells (P < 0.05; Figure 4B). However, the mutation in the predicted binding site of miR-3941 on IGF-1 3’-UTR restored the decreased luciferase activity (P < 0.05; Figure 4B). Moreover, IGF-1 expression in the miR-3941 mimic group significantly decreased compared with that in the scramble and control groups, while it obviously increased in the miR-3941 inhibitor group (P < 0.05; Figure 4C and 4D). These data suggested that IGF-1 was the direct target of miR-3941 and IGF-1 expression was negatively regulated by miR-3941.

miR-3941 overexpression regulated migration, invasion, and EMT-related proteins by targeting IGF-1

To further investigate whether miR-3941 regulated the breast cancer cell migration and
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Figure 3. The effects of miR-935 on cell migration and invasion. A and B: Transwell assay showing the number of migrated cells in different transfection groups. C and D: Transwell assay showing the number of invaded cells in different transfection groups. E and F: The expression levels of E-cadherin, N-cadherin, and vimentin in different groups determined by qRT-PCR and western blot. Error bars indicate means ± SD. *, P < 0.05 and **, P < 0.01 (compared with control group).

invasion by targeting IGF-1, we knocked down IGF-1 using IGF-1-specific siRNA (si-IGF-1). As shown in Figure 5A and 5B, the expression of IGF-1 significantly decreased after si-IGF-1 transfection compared with that in the control group (P < 0.01), indicating that IGF-1 was suc-
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Figure 4. IGF1 was a direct target of miR-3941. A: The results predicted by TargetScan (http://www.targetscan.org/). B: Luciferase report assay showing that miR-3941 can directly target the 3'UTR of IGF1. C: The expression of IGF1 in different transfection groups as determined by qRT-PCR. D: The expression of IGF1 in different transfection groups as determined by western blot. Error bars indicate means ± SD. *, P < 0.05 and **, P < 0.01 (compared with the control group).

cessfully knocked down. In addition, we found that the inhibitory effects of miR-3941 overexpression on cell migration and invasion were reversed when cells were co-transfected with the miR-3941 mimic and si-IGF-1 (P < 0.05; Figure 5C-F). Moreover, the increased expression of E-cadherin and decreased expression of N-cadherin and vimentin, induced by miR-3941 overexpression, were significantly reversed when cells were co-transfected with the miR-3941 mimic and si-IGF-1 (P < 0.05; Figure 5G and 5H). Besides, the effects of miR-3941 suppression on cell migration, cell invasion, and the EMT-related proteins were also reversed when the cells were co-transfected with the miR-3941 inhibitor and si-IGF-1 (P < 0.05; Figure 5C-H). These data indicated that the overexpression of miR-3941 regulated the migration, invasion, and EMT-related proteins in breast cancer cells, possibly by targeting IGF-1.

Discussion

In the current study, inverse expression of miR-3941 (downregulated) and IGF-1 (upregulated) was observed in breast cancer tissues and cells. The overexpression of miR-3941 significantly inhibited the breast cancer cell viability and suppressed migration and invasion. In addition, IGF-1 was shown to be the target of miR-3941, and IGF-1 expression was negatively regulated by miR-3941. The knockdown of IGF-1 significantly reversed the inhibitory effects of miR-3941 overexpression on cell migration, invasion, and EMT-related proteins. These data imply that the crucial roles of miR-3941 in breast cancer may be due to targeting IGF-1.

In a previous study, IGF-1 was shown to play mitogenic and anti-apoptotic roles in mammary epithelial cells, thus contributing to the breast cancer progression [19]. IGF-1 is a crucial regulator of the cell growth and is widely involved in the initiation and progression of breast cancer [20]. IGF-1 can upregulate cysteine-rich angiogenic inducer 61 (Cyr61) through activation of the protein kinase B (also known as Akt)/phosphoinositide 3-kinase (PI3K) pathway, thus inducing breast cancer cell growth and invasion [21]. IGF-1 can also activate human ether-à-go-go (hEAG) K+ channels and subsequently contribute to the cell proliferation in breast cancer [22]. In addition, IGF-1 is also identified as a key regulator mediating the activation of matrix metalloproteinases (MMPs), thus contributing to an increased invasive potential of breast cancer cells [23]. IGF-1 can also induce the secretion of MMP-2, thereby contributing to the cell invasion and angiogenesis of breast cancer cells [24]. In our study,
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A

Relative IGF1 mRNA expression

Control  si-IGF1

B

IGF1
GAPDH

Control  si-IGF1

C

Number of migrated cells/field

Control  Mimic  Mimic+si-IGF1  Inhibitor  Inhibitor+si-IGF1

D

Control  Mimic  Mimic+si-IGF1

Inhibitor  Inhibitor+si-IGF1

E

Number of invaded cells/field

Control  Mimic  Mimic+si-IGF1  Inhibitor  Inhibitor+si-IGF1

F

Control  Mimic  Mimic+si-IGF1

Inhibitor  Inhibitor+si-IGF1

G

Relative mRNA expression

E-cadherin  N-cadherin  Vimentin

Control  Mimic  Mimic+si-IGF1  Inhibitor  Inhibitor+si-IGF1

H

E-cadherin  N-cadherin  vimentin  GAPDH

Control  Mimic  Mimic+si-IGF1  Inhibitor  Inhibitor+si-IGF1
IGF-1 was found to be the target of miR-3941, and IGF-1 expression was negatively regulated by miR-3941. The overexpression of miR-3941 significantly inhibited the breast cancer cell viability and suppressed migration and invasion. Although the roles of miR-3941 in breast cancer, as well as the relationship between miR-3941 and IGF-1, have not been reported previously, our results prompt us to speculate that miR-3941 may inhibit the viability, migration, and invasion of breast cancer cells via targeting IGF-1.

Furthermore, EMT is a crucial event in cancer progression and metastasis [25]. EMT markers such as E-cadherin [26], N-cadherin [27], and vimentin [28] have been found to regulate the invasion and metastasis of breast cancer. In addition, it has been reported that IGF-1 can induce the activation of the latent transforming growth factor beta 1 (TGF-β1), thereby causing and promoting the invasion of breast cancer [23]. CCN6 (WISP3), a cysteine-rich secreted protein, decreases the zinc finger E-box-binding homeobox 1 (ZEB1)-mediated EMT and invasion in breast cancer via suppression of IGF-1 receptor signaling [29]. IGF-1 can enhance the mortality risk in women with breast cancer through regulating EMT [30]. In our study, the expression of E-cadherin was significantly upregulated after miR-3941 overexpression in comparison with that in the scramble group or control group, while the expression of N-cadherin and vimentin was downregulated. In addition, the knockdown of IGF-1 significantly reversed the inhibitory effects of miR-3941 overexpression on cell migration, invasion, and EMT-related proteins. Thus, we speculate that miR-3941 may play vital roles in regulating migration, invasion, and EMT-related proteins in breast cancer cells, possibly by targeting IGF-1. Evidence from clinical evaluation has revealed that increased IGF-1 levels are strongly correlated with a high risk and poor outcome of breast cancer [31, 32], implying the clinical significance of miR-3941 and IGF-1. Further studies are still required to confirm our findings.

In conclusion, our findings reveal that miR-3941 is downregulated in breast cancer cells, and downregulation of miR-3941 may promote the breast cancer cell proliferation, migration, and invasion through not targeting IGF-1. miR-3941 and IGF-1 may serve as diagnostic markers or potential targets for breast cancer treatment.

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Disclosure of conflict of interest

None.

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