

Original Article

The effects of miR-218 on mTOR/AKT signaling and breast cancer cell proliferation

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Abstract: Breast cancer has become the most common female malignant tumor, and microRNA (miR) plays crucial roles in regulating the cell behaviors of malignant tumors. This study examines the targeted effect of miR-218 on the mTOR/AKT signal pathway and cell proliferation in breast cancer. Methods: A gene chip assay was performed to measure the miR expression profile of the breast cancer cell lines BT474, HTB-106, and MCF7. RT-PCR was used to confirm the miR-218 expression. Bioinformatics prediction and a luciferase reporter gene assay were used to confirm the functional target of miR-218. The mimics approach was used to modulate miR-218 expression, and Western-blot quantified the mTOR and p-AKT expressions. A clonal formation assay was employed to analyze the cell proliferation potency. Results: Gene chip and RT-PCR showed suppressed miR-218 expression in the breast cancer cell line ($p < 0.05$). The bioinformatics prediction and the luciferase-reported gene analysis indicated RICTOR as a possible target gene of miR-218. The cell transfection of the mimic elevated the miR-218 expression and remarkably suppressed the RICTOR expression ($p < 0.05$). mTOR and the total Akt protein expression did not have any significant changes, but activated Akt protein showed a decrease ($p < 0.05$). A clonal formation assay indicated that miR-218 could inhibit cell proliferation ($p < 0.05$). Compared to normal breast gland cells, the cancer cells showed a remarkably decreased miR-218 expression. Conclusion: By facilitating miR-218 expression, the RICTOR gene expression was elevated, further suppressing the proliferation potency of the breast cancer cells.

Keywords: Breast cancer, MiR-218, RICTOR, cell proliferation

Introduction

Breast cancer is the most common female malignant tumor and makes up about 29% of all female cancers [1]. The main treatment approaches for breast cancer include chemo- or radio-therapy, surgery, and hormonal treatment. Currently surgery is still the primary approach in treating breast cancer [2]. However, various studies have shown that when breast cancer develops into a 1 cm tumor, it frequently has become a systemic disease with multiple, distal small metastatic lesions [3]. Chemotherapy can manage the focal recurrence of breast cancer to a certain extent. However, the occurrence of drug resistance largely compromises the treatment efficiency of breast cancer [3, 4]. Therefore, further investigation of the regulatory mechanism for breast cancer cell proliferation, and the suppression of breast

cancer cell division and metastasis at the cellular level, are of critical importance for improving treatment efficiency and decreasing medical burdens.

A previous study showed the critical roles of the AKT-mTOR signal pathway in various cell processes, including proliferation, apoptosis, cancer pathogenesis, and drug resistance [5]. When exogenous growth factor or cytokine binds with the cell surface receptor, PI3K upstream from the mTOR pathway can be recruited and activated, further initiating Akt molecules. As one protein kinase, activated Akt protein can facilitate cell proliferation and division. Previous studies showed that the abnormal activation of the AKT-mTOR signal pathway is related to multiple cancers, including lung cancer, liver cancer, and gastric carcinoma [6-8].

Table 1. qRT-PCR primer sequences

Name	Sequence
miR-218-F	5'-GATCCGACCAGTCGCTGCGG-3'
miR-218-R	5'-CAAGCTTTGCAGGAGAGCAC-3'
U6-F	5'-CTCGCTTCGGCAGCAC-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'

miR-218 is one non-coding small RNA molecule belonging to the miR family. Previous studies showed that miR can modulate multiple cell behaviors through the RNA interference mechanism [9, 10]. By analyzing the expression and function of miR in various malignant tumor cells, researchers concluded that miR plays important roles in mediating malignant tumor cells. Previous studies found abnormal expression levels of miR-218 in breast cancer cell lines, and its possible involvement in cancer cell proliferation modulation [11, 12]. This study aimed to investigate the effects of miR-218 on the AKT-mTOR signal pathway of breast cancer cells and its function in cancer cell proliferation.

Materials and methods

Materials

The breast cancer cell lines BT474, HTB-106 and MCF7, along with the normal breast gland epithelial cell line HBL-1000, were purchased from the Chinese Medical Academy. The DMEM cell culture medium, penicillin, and streptomycin were purchased from Gibco (US). The RNA extraction kit was purchased from Qiagen (US). The improved culture medium was purchased from Invitrogen (US). The miRNA4.0 assay chip was purchased from Affymetrix (US). The mirVanat qRT-PCR miRNA test kit was purchased from Ambion (US). The real-time PCR cyclers were purchased from Bio-Rad (US).

Cell culture

The breast cancer cells were incubated in DMEM medium (15% fetal bovine serum, 100 u/ml penicillin, and 100 µg/ml streptomycin). The HBL-100 cell line was maintained in a DMEM medium (containing 10% fetal bovine serum, 100 u/ml penicillin, and 100 µg/ml streptomycin). After 3 days of cell culture, the cells were passed. The culture medium was removed from the flask, and the cells were

digested in trypsin, and then they were re-suspended in a culture medium. The cells were then inoculated into a fresh medium in the amount of 10^5 at 37°C with 5% CO₂.

miRNA expressional profile assay

Cultured breast cancer cells were extracted for total RNA using an RNeasy pure Tissue Kit (QIAGEN, US). A FlashTag Biotin HSR kit (Affymetrix, US) was used to add tails and biotin labels on the miRNA of the total RNA samples. Labelled samples were rinsed with a miRNA 4.0 chip and were scanned in a GCS3000 analyzer (Affymetrix). The raw data were input into ExpressoinConsole software (Affymetrix) to subtract the background. The average values of the replicates and standard errors were calculated and were normalized. Using an independent sample test, statistical significance was defined as $p < 0.01$.

qRT-PCR

First, qRT-PCR primers were designed based on the miR-218 sequence (GeneBank access No. NR_029631) as shown in **Table 1**. Using total RNA extracted from the HBL-100 cell line as the control, qRT-PCR was employed to measure the miR-218 expression level in the breast cancer cells. A mirVanat qRT-PCR miRNA test kit (Ambion) was used for qRT-PCR on a cycler (Bio-Rad) under the following conditions: 95°C for 3 min, followed by 40 cycles consisting of 95°C for 15 s, and 60°C for 30 s. The built-in software V 2.02 was used for the data analysis. Using the U6 gene sequence as the internal reference, the results were presented using the $2^{-\Delta\Delta Ct}$ approach [13].

miR-218 functional prediction

Bioinformatics software TargetScan Release 5.1 (www.targetscan.org) was used to predict the miR-218 function. A luciferase reporter gene assay was used to confirm the possible targets of miR-218. Based on the 3'-UTR of RICTOR mRNA sequence (Genbank access No. AY515854), these primers were designed: (5'-TATAT CTAGA TTCTT GTTAT TACGC TGTTT TG-3'; 5'-AGATT CTAGA ATGTC ATATA CCAAT ATGGC-3'). The 3'UTR sequence of RICTOR mRNA was amplified by PCR and was inserted into the downstream of the firefly luciferase gene coding region within a pmirGLO vector to

construct the pmirGLO-RICTOR plasmid. Using HEK293 as the cell model, the pmirGLO-RICTOR and empty pmirGLO plasmids were transfected, and the cells with successful transfections were double transfected with the miR-218 mimic to elevate the miR-218 levels in the live cells as described below. The cells with successful transfections were cultured for 48 h for a fluorescent intensity analysis. Using the dual luciferase reporter gene assay system (Promega) and MicroLumatPlus LB96V spectrometry (Berthold), the fluorescent intensity was analyzed [14].

Cell transfection of miR-218

The MiR-218 mimic and the miR-218 inhibitor were used to elevate or suppress the miR-218 expression in the breast cancer cell line. The miR-218 mimic and the miR-218 inhibitor were purchased from Gimma Pharm (China). The parallel negative control was performed using a sequence similar to the miR-218 mimic and the miR-218 inhibitor. A liposome INTERFER in transfection kit (Polyplus transfection) was used for the cell transfection assay. The cells were resuscitated and cultured until they reached the log-growth phase, and then they were digested in trypsin, counted, diluted in a fresh medium, and then they were inoculated into a 96-well plate. After 24 h culture, the transfection was performed according to the transfection kit's instruction manual.

Cell clonal formation assay

After they were transfected with miR-218 mimic and miR-218 inhibitor, the breast cancer cells were cultured until the log-growth phase, and then they were digested in trypsin and resuspended in a fresh medium. The cell suspension was inoculated into a culture plate containing 10 mL fresh medium at 100 cells per well density, and then they were gently swirled for an even distribution. The processing of each group of cells was performed in triplicate. The cells were inoculated for 10 d in a chamber, and the supernatant was discarded. The cells were rinsed in PBS, and then they were fixed in 2% paraformaldehyde for 15 min. The cells were then stained in 5% crystal violet for 15 min. The staining buffer was discarded, and the dish was inverted and placed on a transparent film with mesh imprinted for calculating the number of

clones. Clonal formation rate = (clone number/100) × 100%.

Flow cytometry analysis

Flow cytometry was used to measure the cell proliferation potency of breast cancer cells. Using untreated breast cancer cells as a blank control, all the cells were cultured to the log-growth phase. The culture medium was removed, and the cells were rinsed in PBS, and then they were fixed in 90% ethanol overnight. The ethanol was then removed and RNase was added at 37°C for 30 min. A PI solution was used for the cell staining. A flow cytometry apparatus (Becton Dickinson) was used for the analysis using the 488 nm excitation wavelength and the 630 emission wavelength. 10,000 fluorescent signals were collected from each sample, and Modifit software was used to analyze the FL-2 area and the DNA histogram. All experiments were performed in triplicate, and the percentages of cells at specific cycles were calculated [13].

Statistical analysis

All data were analyzed for variance, and results were presented as the mean ± standard deviation (SD). SPSS 20.0 software was used for Student's *t*-test. A significance was defined as $p < 0.05$, and extreme significance was defined as $p < 0.01$.

Experimental results

Differential expression of miRNA in breast cancer cells

Total RNA was extracted from breast cancer and HBL-100 cells. A gene chip was used to measure miRNA expression in the cancer and control cells in triplicate. Expression Console software was used to analyze the miRNA expression level in the breast cancer cells, using the miRNA expression in the HBL-100 cells as the reference. As shown in **Figure 1**, the breast cancer cells showed 35 miRNA molecules with significantly altered expressions compared to the HBL-100 cells, including the up-regulation of miR-214, miR-127, miR-155, and miR-338, plus the prominent down-regulation of miR-429, miR-200a, and miR-218 ($p < 0.05$).

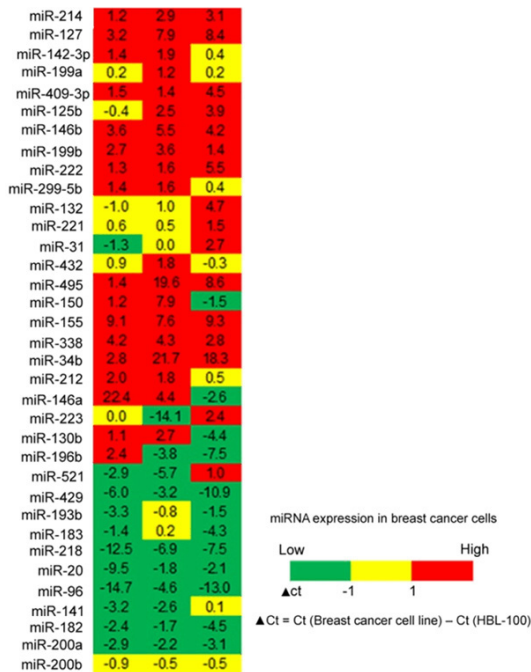


Figure 1. MiRNA expression in breast cancer cells by gene chip assay.

RT-PCR for miR-218 expression

RT-PCR was employed to confirm the result from a gene chip assay. Using U6 RNA as the internal reference, the relative expression of miR-218 was calculated. As shown in **Figure 2**, the miR-218 expression level in breast cancer cells was significantly lower than it was in the HBL-100 cells, consistent with gene chip assay result ($p < 0.01$).

Relationship between miR-218 and the RICTOR gene

Bioinformatics software TargetScan Release 5.1 was used to predict the target genes of miR-218. We found a certain sequence homology between miR-218 and 3'UTR of RICTOR (**Figure 3A**). Therefore, it is speculated that the RICTOR gene might be a target gene of miR-218. We thus constructed a luciferase reporter gene expression system for substantiation. As shown in **Figure 3B**, we found a remarkably decreased cell fluorescence intensity after transfecting the miR-218 mimic, while the miR-218 inhibitor transfection showed a remarkably elevated cell fluorescence intensity. These results showed that the 3'UTR of RICTOR was a functional target of miR-218.

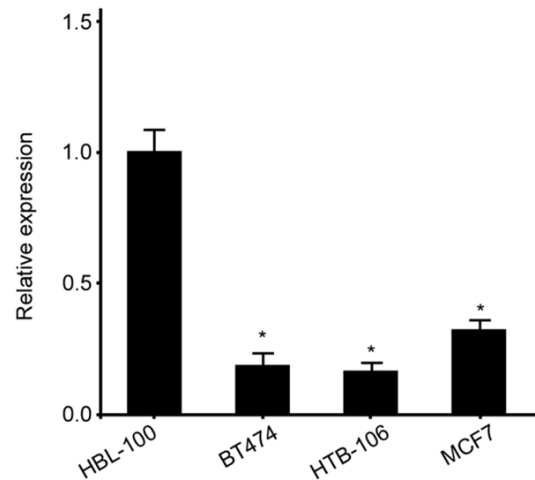


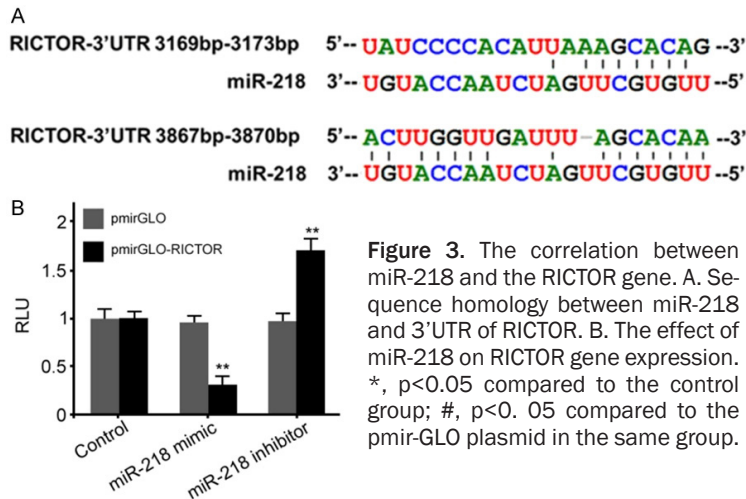
Figure 2. Relative expression in breast cancer cells by RT-PCR. *, $p < 0.01$ compared to HBL-100 cells.

The effects of miR-218 on RICTOR gene expression in breast cancer cells

Using the miR mimic and inhibitor transfection approaches, we specifically over-expressed or inhibited miR-218 in breast cancer cells. The transfected cells were cultured until they reached the log-growth phase, and total RNA was extracted from the cultured cells to measure miR-218 relative expression using RT-PCR. Western Blot was used to measure the RICTOR expression level. As shown in **Figure 4**, after the transfection of the miR-218 mimic, the expression level of miR-218 in breast cancer cells was significantly up-regulated ($p < 0.05$), while the transfection miR-218 inhibitor remarkably suppressed miR-218 expression ($p < 0.05$). These results indicated the change of microRNA expression as predicted. We also found that breast cancer cells with the miR-218 mimic transfection showed a significant down-regulation of RICTOR ($p < 0.05$), and cells with the miR-218 inhibitor transfection presented a remarkably elevated RICTOR expression ($p < 0.05$).

Change of breast cancer cell proliferation

A clonal formation assay was used to measure the proliferation potency of breast cancer cells with the miR-218 mimic and miR-218 inhibitor transfections. As shown in **Figure 5**, we found suppressed clonal formation potency in breast cancer cells with the miR-218 mimic transfection ($p < 0.05$), and the breast cancer cells with an miR-218 inhibitor transfection showed an



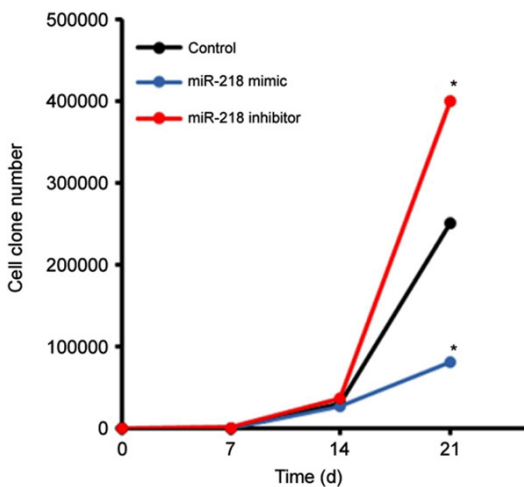
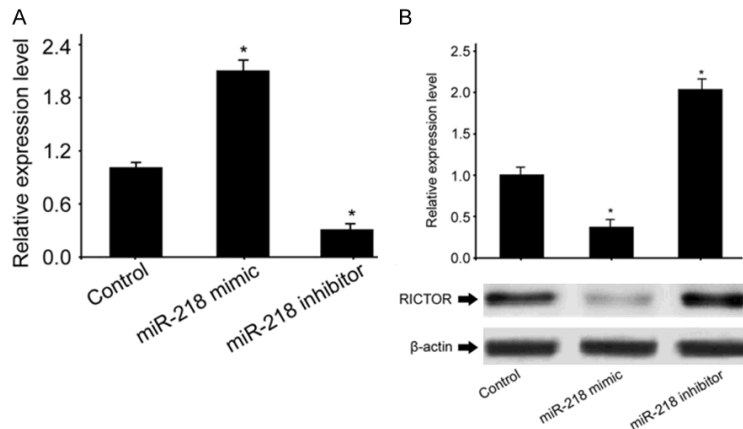
elevated clonal formation potency (p<0.05). These results indicated that miR-218 could inhibit the proliferation of breast cancer cells.

The effects of miR-218 on mTOR signal pathway protein expression

Using Western blot, we measured the effects of miR-218 on the mTOR signal pathway. As shown in **Figure 6**, compared to the control group, the breast cancer cells transfected with miR-218 showed no significant change of mTOR or total Akt proteins, but the phosphorylated Akt protein expression level was significantly decreased (p<0.05).

Discussion

Several recent studies have demonstrated a major difference in microRNA expressions between breast cancer cells and normal cells, including miR-21, miR-10b, and miR-218 [15-17]. Breast cancer cells can modulate cell proliferation and differentiation by regulating microRNA expression. For example, miR-10b participates in breast cancer cell invasion and metastasis [15]. MiR-218 also plays



an important role in the proliferation of breast cancer cells [12]. Other studies showed that microRNA expression is closely correlated with the drug resistance of breast cancer cells [14]. This study first analyzed the difference of the microRNA expression profiles between breast cancer cell lines and the normal breast gland epithelial cell line HBL-100 and found the down-regulation of multiple miRNA molecules including miR-218. We then used bioinformatics prediction and a luciferase reporter gene assay plus cell transfection, and we confirmed that the miR-218 functional target was RICTOR. Lastly, we performed a cell clonal assay and Western blot to demonstrate that miR-218 could inhibit breast cancer cell proliferation by modulating the mTOR signal protein expression.

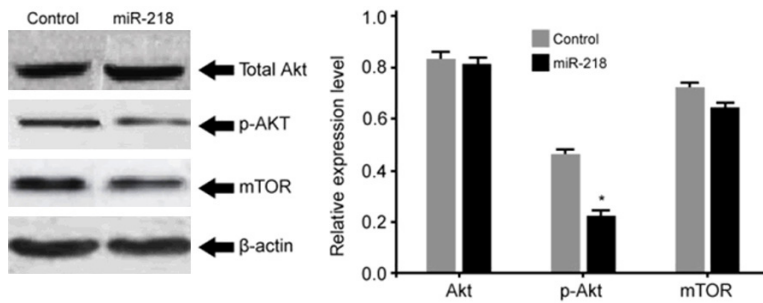


Figure 6. The effects of miR-218 on mTOR pathway protein expression in breast cancer cells. *, $p < 0.05$ compared to the control group.

Several recent studies showed a down-regulation of miRNA expression in breast cancer cells. However, how malignant tumor cells achieve a differential expression of microRNA is still debatable [18, 19]. Previous studies showed that when using a demethylation reagent to treat tumor cells, parts of the microRNA showed up-regulation. Therefore, some researchers found that the expressional alternation of miR in malignant tumor cells was due to methylation or some other type of irreversible modification of its transcriptional regulatory sequence [18]. Ausushi et al. investigated the DNA methylation level of miR-218 and miR-585 sequences in oral squamous carcinoma cells and found decreases in miR-218 and miR-585 by 73.3% and 66.7%, respectively, in oral squamous carcinoma cells. Meanwhile, DNA the sequence of miR-218 and miR-585 showed significant methylation levels compared to normal cells [19].

Within the Akt/mTOR signal pathway transduction, when exogenous growth factors and cytokine binds with the cell surface receptors, PI3K at the upstream of the mTOR pathway can be recruited and activated for further activation of the Akt molecule. As one well-known protein kinase, Akt has important effects on cell proliferation and division [8]. A previous study found that activated Akt protein can further activate mTOR [8]. Such activated mTOR can modulate cell proliferation and apoptosis by phosphorylating certain factors during protein translation, such as 4EBP1 and P70S6K [20, 21]. RICTOR is one characteristic scaffold protein of mTORC2, and is required for exerting the kinase activity of mTOR. A recent study showed important roles of RICTOR in tumor progression, and RICTOR down-regulation can inhibit the tumor lesion formation of a mouse xenograft assay and the proliferation of human prostate cancer

cells [22]. This study found that RICTOR inhibition by miR-218 up-regulation suppresses the proliferation potency of breast cancer cells, which is consistent with other studies.

As the most common female malignant tumor, breast cancer severely threatens women health [1]. Breast cancer cells mainly achieve recurrence, metastasis, and drug resistance

via cancer stem cells, thus making the suppression of breast cancer stem cells of critical importance in cancer treatment. This study found a significant down-regulation of miR-218 in breast cancer cells and found that cancer cells have facilitated the proliferation and carcinogenesis potency by suppressing miR-218 expression to enhance RICTOR gene expression. This may provide new insights for the clinical treatment of breast cancer. Using a molecular biological approach to elevate miR-218 expression in breast cancer stem cells, cell proliferation and recurrence can be inhibited. However, the exact mechanism by which miR-218 is involved in the regulation of cell proliferation of breast cancer cells remains unclear and requires further investigation.

Conclusion

Compared to normal breast gland cells, cancer cells show a significant down-regulation of miR-218. By suppressing miR-218 expression within breast cancer cells to inhibit the RICTOR gene, the proliferation of breast cancer cells can be inhibited.

Disclosure of conflict of interest

None.

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