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Effect miR-214-3p on proliferation and apoptosis of breast cancer cells by targeting survivin protein

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Abstract. – OBJECTIVE: Studies showed that microRNAs (miRs) play an important role in the development of breast cancer. It has been shown that there were significant differences between the expression levels of serum miR-214-3p in breast cancer patients and healthy controls. Since survivin is involved in cell cycle and apoptosis, this study aims to investigate the effect of miR-214-3p on the proliferation and apoptosis of breast cancer cells.

MATERIALS AND METHODS: Dual-Luciferase reporter system was used to validate the cell cycle-related target gene survivin. miRanda and TargetScan were used to predict miR-214-3p target genes. Lipofectamine 2000 was used to transfect the miR-214-3p mimics, miR-NC into the MCF-7 cells. The quantitative Real Time-PCR (qRT-PCR) was used to detect the expression levels of miR-214-3p and survivin. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to examine the cell proliferation of breast cancer cells. The flow cytometry assay was used to evaluate the apoptosis of breast cancer cells.

RESULTS: Dual-Luciferase reporter assay showed that cells co-transfected with wild-type vector and miR-214-3p mimics had significant lower ratios of hRluc/Luc fluorescence compared to that of the control group ($p<0.05$). The expression level of miR-214-3p was increased along with the increase of time after transfection, whereas the expression level of survivin mRNA was decreased along with the increase of time post transfection. This result suggests that miR-214-3p regulates the mRNA expression of survivin. Transfection of miR-214-3p inhibitor increased the proliferation of MCF-7 cells and transfection of miR-214-3p mimics decreased the proliferation of MCF-7 cells compared to control group ($p<0.05$).

CONCLUSIONS: Survivin gene is a downstream target of miR-214-3p in breast cancer cells. The expression of miR-214-3p and survivin is correlated with the proliferation and apoptosis of breast cancer cells.

Key Words:
MiR-214-3p, MCF-7 cells, Survivin, Proliferation, Apoptosis.

Introduction

Breast cancer is a common cancer in women. Published data show that breast cancer ranks first among new female cancer[1]. MicroRNAs (miRs) are non-coding RNA with a length of about 19 to 24 nucleotides, leading to altered expression of target genes by binding to targeted mRNA 3'UTR to degrade mRNA or suppress transcription. miRNAs play an important role in normal embryonic development, cell proliferation, and cell metabolism[2]. Studies[3,4] have shown that abnormal expression of miRNAs is associated with a variety of cancers. Single miRNA may be combined with more than 100 mRNAs to transcriptionally regulate their expression to control cell growth, differentiation, and apoptosis. It has been shown that miR-122 affected the growth of breast cancer cells by regulating the expression of the ARH1[5], miR-204 regulated apoptosis by targeting the JAK2/STAT3/BCl-2/surviving[6]. In breast cancer, the majority of miRNAs were differentially expressed. It has been shown that miR-10b and miR-125b play an important role in the development, metastasis, and invasion of breast cancer[7]. miRNA miR-214-3p is a miRNA which can suppress tumor growth and metastasis by inhibiting the expression of LZTS1[8]. However, the exact role of miR-214-3p in breast cancer remains unclear. Survivin is an apoptosis-related factor which has been shown to inhibit cell death and promote angiogenesis in tumorigenesis[9,10].

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Material and Methods

Main Instruments and Cell Culture

TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Real-time PCR detection kit was purchased from TaKaRa (Biotechnology Co., Ltd., Dalian, China). ABI 7500 fluorescence quantitative PCR instrument was purchased from ABI (Foster City, CA, USA). Dual-Luciferase reporter gene assay kit was purchased from Promega (Madison, WI, USA). MiR-214-3p mimics, miR-214-3p inhibitor, and miR-normal control (miR-NC) mimics were purchased from Guangzhou RuiBo Biosciences (Guangzhou, China). Flow cytometer was purchased from Beckman Coulter Inc. (Brea, CA, USA). Human breast cancer cell line MCF-7 cells were cultured with Roswell Park Memorial Institute-1640 (RPMI-640) medium containing 10% fetal bovine serum (FBS) in 37°C incubator supplied with 5% CO_2. This research was approved by the Ethical Committee of Tangshan People's Hospital, Tangshan, China.

MiR-214-3p Target Gene Prediction and Luciferase Reporter Assay

miRanda and TargetScan were used to predict the miR-214-3p target genes. According to prediction results, wild-type miR-214-3p targeting survivin sequence and mutant miR-214-3p targeting survivin sequence were synthesized and inserted into down-stream of *Renilla* luciferase gene (hRluc) in priR-RB-Report™ plasmids. The firefly luciferase gene (hLuc) was used as a reference.

Cell Transfection

Cells in the logarithmic growth phase were seeded into 6-well plates and cultured for 2 days. MiR-214-3p mimics or miR-NC, and wild-type or mutant target sequence were co-transfected into cells using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The cells were divided into four groups. Group A: wild-type target sequence and miR-NC co-transfection. Group B: wild-type target sequence and miR-214-3p mimics co-transfection. Group C: mutant target sequence and miR-NC co-transfection; D group: mutant target sequence and miR-214-3p mimics co-transfection. Dual-Luciferase luminescent detector was used quantify the fluorescence. hRluc/hLuc was used for calibration.

RNA Extraction and Quantitative PCR Detection of MiR-214-3p-3p and Survivin

TRIzol reagent was used for the extraction of RNA from MCF-7 cells according to the manufacturer's instruction. Agarose gel electrophoresis showed three bands suggesting that RNA purity and integrity were good. Reverse transcription kit was used to reverse transcribed miRNA or mRNA to cDNA using the ABI 7500 PCR system with the following profile: an initial 30 s incubation at 95°C, 40 cycles of (30 s at 95°C, 60 s at 55°C, and 60 s at 72°C), and a final extension of 3 min at 72°C. The reaction mixtures contained the following ingredients: 2 × SYBR Green Mixture 9 µl, Primer 1 (5 µM) 2 µl, Primer 2 (5 µM) 2 µl, DNA 2 µl, ddH_2O 5 µl. U6 and β-actin were used as reference genes. Each investigation was repeated 3 times and the $2^{-\Delta\Delta CT}$ method was used for the calculation. The primer sequences used in this work can be found in Table I.

3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay of Proliferation

Cells in logarithmic growth phase were seeded in 96-well plates. MTT solution was added to cells at different time points after transfection (24, 48, 72, and 96 h), mixed, and incubated at 37°C in an incubator for 4 h. After withdrawing the supernatant, dimethyl sulfoxide (DMSO) was

Table I. Primer sequences.

Gene	Primer sequences	Amplicon
MiR-214-3p	F: 5'-GCACAGCAGGCACAGACA-3' R: 5'-CAGAGCAGGGTCAGCGGTA-3'	66 pb
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'	83 pb
β-actin	F: 5'-AAACTGGAACGGTGAAGGTG-3' R: 5'-AGTGGGGTGGCTTTTAGGAT-3'	119 pb
Survivin	F: 5'-AGAACTGGCCCTTCTTGGAGG-3' R: 5'-CTTTTTATGTTCCTCTATGGGGTC-3'	126 pb

added to each well, and plates were shaking for 10 min to stop the reaction. Absorbance values at 490 nm were recorded with a microplate reader. Each investigation was repeated three times.

Flow Cytometry Assay of Apoptosis

Cells in the logarithmic growth phase were seeded in 6-well plates. Cells were transfected 24 h after inoculation. Apoptosis was measured at 24 and 48 h after transfection according to the kit instructions. The cells were washed with cold phosphate-buffered saline (PBS), centrifuged at 1000 r/min, resuspended with 300 µl binding buffer, mixed with Annexin V, and incubated at room temperature for 15 min. Propidium iodide (PI) was added 5 min before the measurement.

Statistical Analysis

SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation. The Student's *t*-test was used to compare the differences between the two groups. The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was considered as statistically significant.

Results

Survivin is a Downstream Target Gene of MiR-214-3p

Dual-Luciferase reporter assay results showed that hRluc Group/Luc fluorescence ratio of cells co-transfected with wild-type target sequence and miR-214-3p mimics was significantly lower than that of control group (miR-NC group) ($p<0.05$). No significant difference was found in the hRluc Group/Luc fluorescence ratio of cells co-transfected with the mutant target sequence, miR-214-3p mimics, and cells co-transfected with mutant target sequence and miR-NC (Figure 1), suggesting that survivin is a downstream target gene of miR-214-3p.

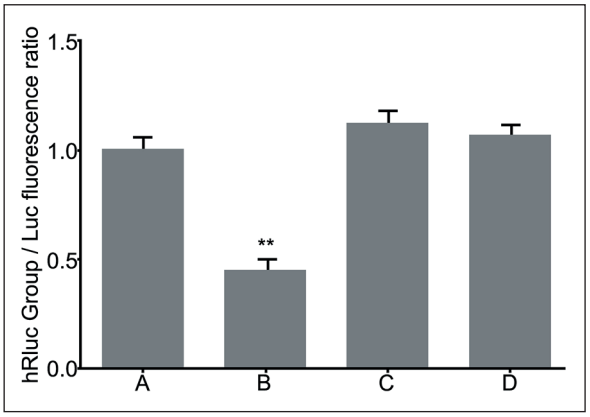

Figure 1. hRluc/Luc fluorescence ratios of different transfection groups shown by Dual-Luciferase reporter assay. Group A: The wild-type target sequence and miR-NC co-transfection. Group B: Wild-type target sequence and miR-214-3p mimics co-transfection. Group C: Mutant target sequence and miR-NC co-transfection. Group D: Mutant target sequence and miR-214-3p mimics co-transfection. $^{**}p<0.05$, compared with group A.

Expression Levels of MiRNA-214-3p and Survivin in MCF-7 Cells

MiR-214-3p mimics or inhibitor were transfected into MCF-7 cells, and the expression levels of miRNA-214-3p and surviving at different time points were measured (Figure 2). The expression of miRNA-214-3p was increased along with the extension of transfection time in cells transfected

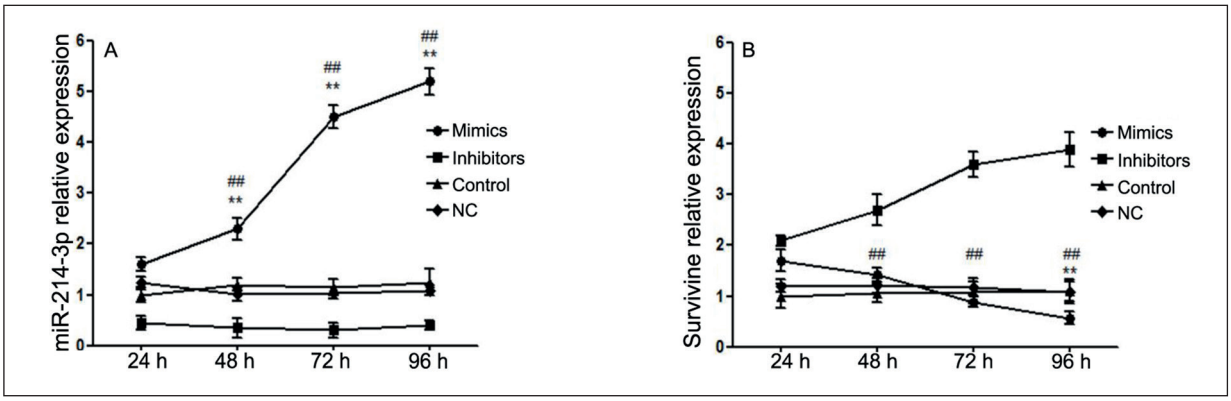

Figure 2. Expression levels of miRNA-214-3p and survivin in MCF-7 cells. ***A,*** Expression levels of miRNA-214-3p. ***B,*** Expression levels of survivin. $^{**}p<0.05$, compared with control group. $^{\#\#}p<0.05$, compared with miRNA-214-3p inhibitor group.

with miRNA-214-3p. The expression of miRNA-214-3p showed no change in cells transfected with miR-NC. The expression of survivin was decreased in cells transfected with miRNA-214-3p mimics, but increased in cells transfected with miRNA-214-3p inhibitor, suggesting that the expression of survivin is regulated by miRNA-214-3p.

Effect of MiR-214-3p Transfection on the Proliferation of MCF-7 Cells

MTT assay was used to measure the proliferation of MCF-7 cells after transfection of miR-214-3p. Results showed that, with the extension of transfection time, the proliferation of MCF-7 cells transfected with miR-214-3p inhibitor was significantly enhanced compared with that of control group ($p<0.05$), suggesting that miR-214-3p may be involved into the process of cell proliferation (Figure 3). Correlation analysis showed a negative correlation between the expression levels of miR-214-3p and cell proliferation ($r=-0.876$) (Figure 4).

Effect of MiR-214-3p Transfection on the Apoptosis of MCF-7 Cells

Flow cytometry was used to measure the apoptosis of MCF-7 cells after the transfection of miR-214-3p. Results showed that, with the extension of transfection time, the apoptosis of MCF-7 cells transfected with miR-214-3p mimics was significantly enhanced compared with that of control group ($p<0.05$), the apoptosis of MCF-7 cells transfected with miR-214-3p inhibitors was significantly suppressed compared with that of control group ($p<0.05$), suggesting that miR-214-3p may be involved in the process of apoptosis (Table II).

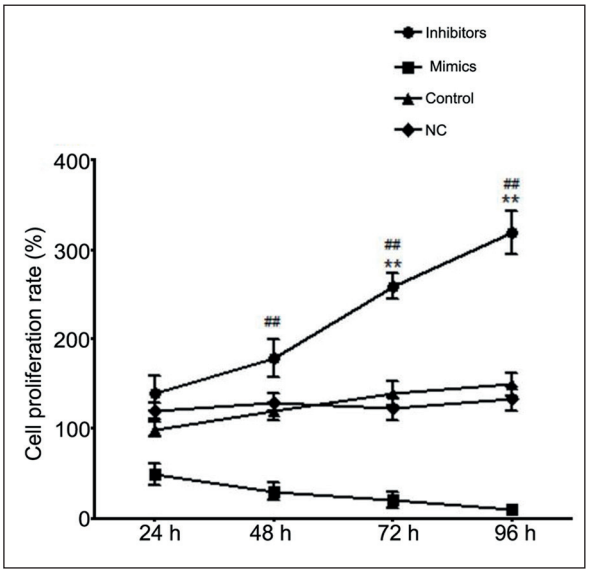

Figure 3. Effect of miR-214-3p transfection on the proliferation of MCF-7 cells. $^{**}p<0.05$, compared with control group, $^{\#\#}p<0.05$, compared with miRNA-214-3p inhibitor group.

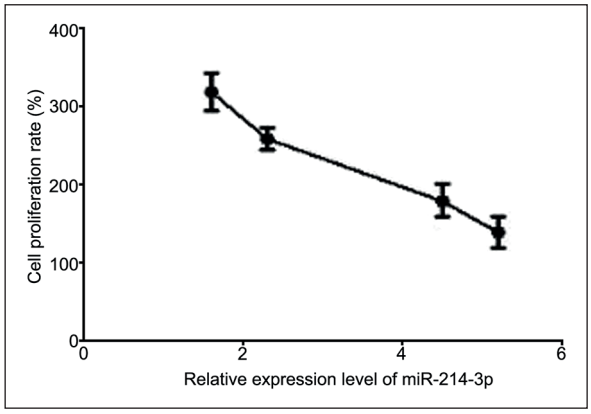

Figure 4. Correlation analysis between the expression levels of miR-214-3p and cell proliferation.

Discussion

In normal cells, miRNAs involved in cell proliferation, differentiation, apoptosis, and other biological functions by the suppression of target genes or degradation of mRNA. Differentially expressed miRNAs may affect normal cell proliferation and differentiation, leading to the occurrence of many types of cancer, cardiovascular, and immune disorders. Reports showed that miRNA expression change plays an important role in tumorigenesis and development. MiRNA microarray analysis of normal breast tissue and breast cancer tissue found that miR-206 could be used as a potential biomarker for the diagnosis of breast cancer[11]. MiR-124-3p expression levels were decreased and promoted the process of breast cancer by targeting Beclin-1[12]. In addition, miR-214 promoted apoptosis of breast cancer cells by targeting RFWD2-p53 and sensitized breast cancer cells to doxorubicin[13]. We showed that[14] miR-214 can target PTEN and PI3K/AKT to participate in the development of breast cancer and affect cell infiltration. The analysis of early oral squamous cell carcinoma samples found that miR-214-3p and miR-375 were of great significance in the prognosis. Briefly, miRNAs play

Table II. Effect of miR-214-3p transfection on the apoptosis rate (%) of MCF-7 cells.

Group	24 h	48 h	72 h	96 h
Inhibitor	3.57 ± 1.25	3.21 ± 3.12**	4.42 ± 2.32**	8.54 ± 3.29**
Mimic	13.23 ± 1.38**	25.43 ± 2.09**	28.69 ± 2.58**	23.87 ± 4.38**
NC	4.37 ± 2.19	8.87 ± 3.34	10.65 ± 2.68	13.18 ± 3.95
Control	5.41 ± 1.59	7.38 ± 1.78	12.47 ± 2.89	13.53 ± 5.15

**$p < 0.05$, compared with control group.

an important role in the malignant proliferation characteristics of breast cancer. Survivin is an important gene involved in apoptosis which is highly expressed in tumor tissue[15]. MiR-214-3p was significantly down-regulated in esophageal squamous cancer cells. By sequence alignment, the 3'UTR of survivin and CUG-BP1 was predicted as its target sequence[16]. However, this needs to be further verified in other cancer cells. This research investigated the role of miR-214-3p in breast cancer cells.

The results of this study showed that miR-214-3p was down-regulated in breast cancer cells, and its target was the cell cycle-related gene survivin, which is consistent with the study of Phatak et al[16]. In esophageal squamous cancer cells, the MTT assay results showed that transfection of miR-214-3p mimics significantly lowered the proliferation level of breast cancer cells compared with that of control group, while transfection of miR-214-3p inhibitor significantly enhanced the proliferation level of breast cancer cells. Liu et al[17] have shown that miR-214 suppresses breast tumor. These results suggested that miR-214-3p inhibits breast cancer. Survivin is an apoptosis-related gene and it mainly inhibits the expression of cysteine-containing aspartate protease 3 and 7 to regulate the cell cycle. At the same time, survivin also is involved in P53-apoptotic pathways[18]. Survivin is highly expressed in the thymus and can be used as a potential molecular marker in the diagnosis of breast cancer [[19]. The down-regulation of survivin showed an anti-tumor effect[20]. Combined with the findings of this work, in the occurrence of breast cancer, the low expression of miR-214-3p resulted in the loss of its control on survivin and thus promoted proliferation and reduced apoptosis. However, due to the differential expression of survivin in different cancers, the specificity may be low. Studies[21,22] also showed that the over-expression of miR-214-3p significantly lowered apoptosis. Besides, we showed that the expression changes of survivin were related to lymphocyte metastasis and colorectal cancer. Therefore, the expression changes of survivin in peripheral blood might be used as a molecular marker for breast cancer diagnosis.

This work is only carried out in cells, but not in the human population. Therefore, whether survivin and miR-214-3p might be used as molecular markers for breast cancer diagnosis needs to be further investigated.

Although this report received some interesting results, there were also a few limitations. First, the present work has not explored the effects of miR-124-3p on breast tumorigenesis, which would be clarified in the following study. Second, miR-124-3p triggered the downregulation of the survivin gene; however, the association between survivin and miR-124-3p has not been clarified.

Conclusions

Survivin may be a downstream target gene of miR-214-3p. The down-regulation of miR-214-3p in breast cancer cells promoted cell proliferation and inhibited apoptosis, suggesting that the down-regulation of miR-214-3p contributed to the development of breast cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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