Original Article
Application of miR-497 in breast cancer and its related mechanisms

Chunhong Xiao¹*, Jianfen Yuan²*, Haizhong Yu², Zhimei Wu², Hong Hong²

¹Department of Clinical Laboratory, Nantong Tumor Hospital, Nantong 226361, Jiangsu, P. R. China; ²Department of Clinical Laboratory, Nantong Traditional Chinese Medicine Hospital, Nantong 226001, Jiangsu, P. R. China.

*Equal contributors.

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Abstract: This study aimed to explore the application and related mechanism of miR-497 in breast cancer (BC). Altogether 107 BC patients who visited our hospital from December 2016 to July 2017 were selected as the study group, and 95 non-BC patients as the control group. Blood and specimens from the two groups of patients were collected. Cell lines MCF-7, MDA-MB-231 and human normal breast cells MCF10A were cultured and transfected, and the expression level of micro RNA-497 was determined by qRT-PCR. CCK8 was used to detect the proliferation, Transwell test to detect the invasion ability, scratch test to detect the migration ability, and the expression difference between p38 mitogen activated protein kinase (p38MAPK) and phosphorylated extracellular signal-regulated kinase (p-ERK) signal protein was detected. Results: The relative expression of plasma miR-497 in the study group was significantly lower than that in the control group, and that in the study group was significantly lower than adjacent tissues. The cancer stage, metastasis, estrogen receptor (ER) and progesterone receptor (PR) are related to the relative expression of miR-497. ROC curve indicates that miR-497 has early diagnostic value for BC (AUC=0.719). The expression level of miR-497 in MCF-7 and MDA-MB-231 cells was significantly lower than that in MCF10A. After transfection, the expression level of miR-497 in the mimic group was significantly higher than that in the other two groups. The number of cell membrane penetrations, migration distance, OD value in each time period and expression of p38MAPK and p-ERK signaling proteins in the mimic group were significantly lower than those in the other two groups. miR-497 is down-regulated in BC, and its expression level is related to the clinical characteristics of BC, which has early diagnostic value for BC. miR-497 affects the proliferation, invasion and migration of BC cells by affecting the expression of p38MAPK and p-ERK signaling proteins.

Keywords: Breast cancer, miRNA-497, application, mechanism

Introduction
Breast cancer (BC) is the most common cancer among women in the world. It is also the second leading cause of cancer death among women, second only to lung cancer [1, 2]. It is predicted that by 2050, the incidence rate of female breast cancer in the world will reach about 3.2 million cases per year, so it is necessary to take preventive and therapeutic measures [3]. In China, due to the rising socioeconomic status and unique birth pattern, China’s proportion to the global fertility rate is rising rapidly. Chinese cases account for 2%-12% of all newly diagnosed BC cases in the world and 6%-9% of all breast cancer deaths in the world [4]. Surgery, radiotherapy, chemotherapy, endocrine therapy, and targeted therapy are the commonly used treatment methods for breast cancer. If relapse, metastasis or drug resistance occurs, the prognosis of patients is often poor [5]. In recent years, the research on cancer mainly focuses on gene diagnosis and treatment, which is helpful for the early detection and treatment of cancer, and is more effective and safe for treatment.

microRNA (miRNA) is a kind of endogenous non-coding RNA molecules, which can be combined with complementary sequences of three major untranslated regions (3-UTR) of target messenger RNA transcripts (mRNAs) to silence genes [6]. miRNAs can regulate the expression of human protein coding genes [7]. Studies show that miRNAs are involved in regulating tumor genesis and drug resistance as oncomiRNAs or tumor suppressors [8, 9]. miRNA-497 (miR-497) belongs to the miRNA200 family...
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[10], which has normal biological functions in organisms. At the same time, its abnormal expression is also involved in influencing the functions of tumor stem cells and various tumors [11-13]. Recent studies show that miR-497 is related to BC [14], but the clinical application of miR-497 in BC and related mechanism research are not clear.

Therefore, through observing the expression of miR-497 in BC patients and its role in BC cells, this study explored its role in BC and related mechanisms, so as to provide reference for clinical practice.

Data and methods

Research subjects

Altogether 107 BC patients who visited our hospital from December 2016 to July 2017 were selected as the study group with an age of (43.25±8.66) years, while 95 non-BC patients who visited our hospital during the same period were selected as the control group with an age of (43.84±8.57) years. Inclusion criteria: females; patients were diagnosed as having primary BC through pathological diagnosis; patients without experience in radiotherapy, chemotherapy or surgery; patients with full clinical data [15]. Exclusion criteria were as follows: patients with severe hepatic and renal insufficiency; patients with serious nervous system diseases; patients with secondary and multiple tumors; and patients without full clinical data. The study was carried out under the permission of the Medical Ethics Committee of our hospital.

The patients and their families were informed by letter or telephone, and they have signed an informed consent form.

Methods

Blood and specimen acquisition: After initial diagnosis of either BC or non-BC, 5 ml of the patients’ peripheral blood was collected and stored in a refrigerator at low temperature, centrifuged at 3,000 rpm for 10 min, and then the serum was frozen. Cancer tissues and adjacent tissues of patients in the study group were obtained, while breast tissue samples were obtained in the control group. After obtaining tissue specimens, necrotic parts, muscles and fat of tumors were removed, rinsed with normal saline, quickly frozen in liquid nitrogen, and then moved to a freezer at -80°C for later use.

Cell culture: Human breast cancer cell lines MCF-7, MDA-MB-231 and human normal breast cells MCF10A were all purchased from ATCC. Cell culture conditions were as follows: RPMI 1640 medium and 10% fetal bovine serum (FBS) (Gibco, USA) were placed in an incubator at 37°C with 5% CO₂ for incubation.

Cell transfection: Breast cancer MCF-7 cells and MDA-MB-231 cells were respectively divided into 3 groups: blank group (without transfection of any plasmid), empty plasmid group (transfection of empty plasmid), and mimic group (transfection of miR-497mimic plasmid).

MCF-7 and MDA-MB-231 cells from a logarithmic growth period were taken for experiments, digested with 0.25% trypsin to form cell suspension, and inoculated into sterile 6-well plates at about 70% density. Fifty ul of serum-free DMEM medium was added into an EP tube, mixed and placed at room temperature for 5 min. Four ul of transfection reagent Hi PerFect Transfection Reagent was added into an EP tube with 50 ul of serum-free DMEM medium. After mixing, it was placed at room temperature for later use. The reagents in the above two EP tubes were mixed together, and the mixture was reversed and placed at room temperature for 20 min. The transfection reaction solution was uniformly added to the target cells at a final concentration of 100 nM, and cultured in serum-free DMEM medium for 72 hours. After the transfected cells were passaged, monoclonal cells were selected by finite dilution method for culture (repeated 3 times). Primers: upstream 5’-CAGCAGCACACUGUGGUUUGU-3’, downstream 5’-AAACCACAGUGUGCUGUUGCU-3’.

Determination of mi RNA-497 expression level by qRT-PCR: Trizol kit was used to extract the total RNA from cells, the specific steps were carried out in strict accordance with the instructions. The concentration and purity of the extracted RNA were detected by UV-3100PC ultraviolet spectrophotometer, and the integrity of the extracted RNA was detected by 1% denatured agarose gel electrophoresis. The extracted RNA was reverse transcribed to obtain cDNA, and the cDNA was used as a template to carry out the experiments. The primer sequence was designed and synthesized by Shanghai Sangon Biotech. In this experiment, β-actin was
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used as an internal reference gene and a reverse transcribed product cDNA was used as a template to amplify on the Light Cycler Real Time PCR amplificator. The reaction system was as follows: 10 μl of SYBRPrimix Ex Taq (2×), 0.4 μl of 5’ and 3’ primers, 2.0 μl of DNA template, and sterilized double distilled water was added to make final volume of 20 μl. The reaction was carried out under the following condition: 95°C for 30 s, 95°C for 5 s, 60°C for 20 s. Melting conditions were as follows: 95°C 0 s, 65°C 15 s, 95°C 0 s. Each group of samples was repeated three times, and the expression level of miR-181c in the samples was analyzed by 2-ΔΔct method. β-actin was used as the internal reference gene, the upstream primer was 5’-5’-GAGACCTTCAA-CACCCCAGC-3’, and the downstream primer was 5’-ATGTCACGCACG-ATTCCC-3’.

Detection of cell migration ability by scratch test: Cells of the NEAT1-siRNA group and Control-siRNA group were cultured in 12-well plates after 24 hours of transfection in each group. After the cells were fully grown and fused, a straight line was drawn by the sterile tips of a pipette with a volume of 20 μl, and the cell repair was observed under microscopes at 0 and 48 h. The scratch healing rate = (scratch area immediately after scratch - scratch area 48 h after scratch)/scratch area immediately after scratch ×100%. The experiment was repeated three times under the same conditions. The migration ability was positively correlated with scratch healing rate.

Detection of MAPK/ERK signal related protein: Total cell protein was extracted from RIPA lysate and quantified by PCA. Sampling, electrophoresis and transmembrane were performed according to the amount of 50 μg/well. After transmembrane, the membrane was blocked using 5% skimmed milk powder for 1.5 h, immersed in TBST for 5 min and then washed. The p38 mitogen activated protein kinase (p38MAPK) and phosphorylated extracellular signal-regulated kinase (p-ERK) protein antibodies were used with reference to the antibody specifications, incubated overnight at 4°C, and the membrane was washed with TBST 3 times, for 10 min each time. ECL was used for developing, and GAPDH was used as internal reference to analyze the relative expression of each index.

Observation indicators

The relationship between miR-497 and clinical characteristics of BC patients and its early diagnostic value were analyzed. The relative expression of miR-497 in plasma and tissues of the two groups were compared. The expression difference of miR-497 in various types of cells, the expression level of miR-497 under various interventions after BC cell transfection, and the effect of miR-497 on BC cell activity, invasion and migration were compared. The effect of miR-497 transfection on MAPK/ERK signal-related proteins in breast cancer cells was analyzed.

Statistical methods

SPSS 19.0 (Asia Analytics Formerly SPSS China) was used. The measurement data were expressed by [n (%)], and comparison of rates
Results

Differences in relative expression of miR-497 between the two groups of patients

The relative expression of plasma miR-497 in the study group was (1.01±0.98) and that in the control group was (1.94±1.69). The relative expression of plasma miR-497 in the study group was significantly lower than that in the control group (P<0.05). The relative expression of miR-497 was (0.69±0.78) in cancer tissues and (1.36±1.13) in adjacent tissues of the study group. Cancer tissues showed remarkably lower relative expression of miR-497 than their adjacent tissues (P<0.05). See Figure 1 for details.

Correlation analysis between miR-497 and clinical characteristics of BC patients

The correlation between miR-497 and clinical features in the study group was analyzed. The results showed that the cancer stage, metastasis, estrogen receptor (ER) and progesterone receptor (PR) were related to the relative expression of miR-497 (P<0.05). See Table 1.

ROC curve analysis of early diagnostic value of miR-497 for BC

The ROC curve was visualized to analyze the early diagnostic value of miR-497 in BC based on the expression of miR-497 in the initial diagnosis of patients. Through detection, it was found that the area under the miR-497 curve was 0.719, the sensitivity was 56.84%, the specificity was 84.11%, the critical value was 1.808, and the 95% CI was 0.647-0.792. See Figure 2.

Expression differences of miR-497 in three cell lines

The expression level of miR-497 in MCF10A cells was taken as a control, and the expression levels in MCF-7, MDA-MB-231, and MCF-10A were compared. The analysis of variance revealed statistically significant expression differences among the three groups (P<0.05). The LSD comparison showed that the expression level of miR-497 in MCF-7 and MDA-MB-231 was significantly lower than that in MCF10A (P<0.05), while the expression level of miR-497 in MCF-7 and MDA-MB-231 was not significantly different (P>0.05). See Table 2.

Comparison of expression levels of miR-497 after transfection

The expression levels of miR-497 in MCF-7 and MDA-MB-231 cells after transfection were ana-
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In MCF-7 and MDA-MB-231 cancer cells, the variance results of the blank group, empty plasmid group and mimic group showed that the expression difference of the three groups was statistically significant (P<0.05). The pairwise comparison of LSD showed that miR-497 expression level in the mimic group was significantly higher than that in the other two groups (P<0.05), while there was no significant difference between the blank group and empty plasmid group (P<0.05). See Table 3.

Effect of miR-497 on BC cell activity

After transfection, the OD values of MCF-7 and MDA-MB-231 cancer cells in the blank group and the empty plasmid group were significantly higher than those in the mimic group at 24, 48 and 72 h (P<0.05). There was no significant difference between the OD values of the blank group and the empty plasmid group at 24, 48 and 72 h (P<0.05). See Figure 3.

Effect of miR-497 on invasion of BC cells

Through Transwell experiments, the invasive ability of the cells was analyzed. The results showed that among the MCF-7 cells, the number of cells passing through microporous membranes of invasive ventricles in blank group, empty plasmid group and mimic group were 384.22±53.32, 375.16±50.14, 146.87±35.19, respectively. Among the MDA-MB-231 cells, the number in blank group, empty plasmid group and mimic group were 423.69±62.39, 409.84±59.62, 152.63±38.15, respectively. The difference among the three groups was statistically significant (P<0.05). The pairwise comparison of LSD showed that the expression level of cell membrane penetration number in the mimic group was significantly lower than that in the other two groups, and there was no significant difference between blank group and empty plasmid group. See Figure 4 for details.

Effect of miR-497 on activity migration of BC cells

The results of the scratch test showed that the migration distances of MCF-7 cells were 84.15±15.65, 79.67±14.33, 42.18±8.12 in blank group, empty plasmid group and mimic group, respectively. The pairwise comparison of LSD showed that miR-497 expression level in the mimic group was significantly lower than that in the other two groups (P<0.05). See Table 3.
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<table>
<thead>
<tr>
<th>miR-497</th>
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<td>MCF-7</td>
<td>0.28797io</td>
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<td>MCF10A</td>
<td>1.000A-23</td>
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group respectively, and 64.32±8.29, 67.56±9.36, 35.28±6.94 in MDA-MB-231 cells. The difference in migration distance among the three groups was statistically significant (P<0.05). The pairwise comparison of LSD showed that the migration distance of cells in mimic group was significantly lower than that in the other two groups (P<0.05). There was no significant difference between blank group and empty plasmid group (P>0.05). See Figure 5 for details.

Effect of miR-497 transfection on MAPK/ERK signal related proteins in BC cells

The results of both cell lines showed that miR-497 had statistically significant difference in the expression of p38MAPK and p-ERK signal proteins of the two cancer cell lines in the three groups (P<0.05). The pairwise comparison of LSD showed that there was no significant difference in the expression of the two signal proteins between the blank group and the empty plasmid group (P>0.05), and the expression of the two signal proteins in the mimic group was significantly lower than that in the other two groups (P<0.05). See Table 4.

Discussion

Breast cancer is one of the most common cancers, with a high incidence rate in all countries. It is the second most common cancer, accounting for 25% of all cancers [16]. Although efforts are being made to prevent the disease, the incidence rate in most countries is still rising and is expected to further rise in the next 20 years [17]. BC is a heterogeneous and complex disease, which is composed of many subtypes and has different biological characteristics, resulting in different response modes to various treatment methods and clinical results [18]. Traditional treatment modes cannot achieve better prognosis. In recent years, gene diagnosis and treatment of cancer is a research direction with more clinical attention. miRNA plays a part in protein coding as a tumor suppressor. Targeted therapy by exploring miRNA mechanisms and targets is a new idea for clinical treatment of cancer [19, 20].

This study analyzed the expression of miR-497 in BC patients and its role in BC cells, so as to explore the application value and mechanism of miR-497 in BC cells. The results showed that the relative expression of plasma miR-497 in the study group was significantly lower than that in the control group, and the relative expression of miR-497 in cancer tissues of the study group was significantly lower than that in adjacent tissues. The cancer stage, metastasis, estrogen receptor (ER) and progesterone receptor (PR) of BC were related to the relative expression of miR-497. ROC curve indicated that miR-497 has diagnostic value for early diagnosis of BC. In terms of expression difference, the expression level of miR-497 in MCF-7 and MDA-MB-231 was significantly lower than that in MCF10A, while the level of miR-497 in MCF-7 and MDA-MB-231 had no obvious difference. After transfection, the expression level of miR-497 in three plasmid groups was significantly higher in the mimic group than in the other two groups, and there was no significant difference between the blank group and empty plasmid group. On the influence of cell activity, the OD values of the blank group and empty plasmid group at 24, 48 and 72 h were significantly higher than those of the mimic group. There was no significant difference of the OD values between the blank group and empty plasmid group at 24, 48 and 72 h. In terms of cell invasion ability, the expression level of cell membrane penetration number in the mimic group was significantly lower than that in the other two groups (P<0.05). In terms of cell migration ability, the cell migration distance in the mimic group was significantly lower than that in the other two groups, and there was no significant difference between the blank group and empty plasmid group. The expression of miR-497 on p38MAPK and p-ERK signaling proteins in the two cancer cell lines was significantly lower in the mimic group than in the other two groups.

Research indicated that miR-497 expression was negatively correlated with SMAD7 expression in BC tissues. Bioinformatics analysis defined a potential miR-497 response element in the 3' untranslated region of SMAD7 and was
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Table 3. Comparison of expression levels of miR-497 after transfection

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<tr>
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<th>Blank group</th>
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<td>1.09MB-23</td>
<td>3.77MB-23</td>
<td>34.640</td>
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Figure 3. Effect of miR-497 on BC cell activity. In MCF-7 and MDA-MB-231 cancer cells, OD values of blank group and empty plasmid group were significantly higher than those of mimic group at 24, 48 and 72 hours. *indicates that compared with mimic group at the same period of time, P<0.05.

Figure 4. Effect of miR-497 on invasion ability of BC cells. In MCF-7 and MDA-MB-231 cancer cells, the expression level of cell membrane number in the mimic group was significantly lower than that in the other two groups. *indicates that P<0.05.

Figure 5. Effect of miR-497 on BC cell migration. In MCF-7 and MDA-MB-231 cancer cells, the cell migration distance in the mimic group was significantly lower than that in the other two groups. *indicates that P<0.05.

verified in gene experiments report. Through MTT and invasive experiments, MDA-MB-231 and MCF-7 BC were inhibited from growing, and flow cytometry was induced to detect S-phase retardation. In addition, up-regulation of miR-497 expression through simulated therapy could significantly inhibit tumor growth in an in-situ nude mouse model, and high expression of
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miR-497 has better prognosis. The research results confirmed the proliferation promotion effect of SMAD7 in breast cancer, thus establishing a regulatory mechanism of SMAD7 in breast cancer through a post-transcriptional mechanism miR-497 [21]. Another study pointed out that in estrogen receptor α (ER-α) BC, miR-497 promotes proliferation, migration and invasion of ER-α negative BC cells by downregulating targeting estrogen related receptor α [22]. The results indicated that the targeting effect of miR-497 on BC is not limited to a single receptor or a single effect. In the role of other cancers, studies have proved for the first time that miR-497 is a regulator of actin binding protein (ANLN) and 70 kDa heat shock protein 4-like protein (HSPA4L). This effective tumor suppressor may lead to increased expression of ANLN and HSPA4L, leading to the development and progress of nasopharyngeal carcinoma [23]. Another study pointed out that the down-regulation of miR-497 in human osteosarcoma can induce apoptosis through PI3K/Akt pathway survival and cisplatin resistance [24]. These studies showed that miR-497 has different action targets and different action mechanisms in different cancers. In addition, miRNA is a new potential biomarker, which can be used for diagnosis, prediction of treatment or prognosis, and is crucial for improving patient management. The main challenge is that these findings need to be firmly validated in more independent cohorts or through more experimental and clinical studies [25].

To sum up, miR-497 is down-regulated in BC, its expression level is related to the clinical characteristics of BC and has early diagnostic value for BC. Its expression level also affects the proliferation, invasion and migration ability of BC cells. miR-497 acts by affecting the expression of p38MAPK and p-ERK signaling proteins in cancer cells.

Disclosure of conflict of interest
None.

Address correspondence to:
Haizhong Yu, Department of Clinical Laboratory, Nantong Traditional Chinese Medicine Hospital, Nantong 226001, Jiangsu, P. R. China. Tel: +86-13390942478; E-mail: yuhaizhong11@126.com

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Table 4. Effect of miR-497 transfection on MAPK/ERK signal related proteins in BC cells

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<td>0.43*1K23</td>
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Note: *indicates that compared with the same cell signal protein mimic group, P<0.05.
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