miR-17-5p Combined with Epirubicin Inhibits the Progression of Breast Cancer Cells

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Research

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Abstract

Background: Breast cancer is the most common malignant tumor for women, which has been ranked first in women's cancer-related death. The objectives of the study are to uncover the underlying mechanisms of combination therapy of epirubicin with miR-17-5p in breast cancer.

Methods: The expression levels of miR-17-5p were determined by quantitative RT-PCR. The survival rate of MCF-7 cell was detected respectively by MTT assay. The expression levels of miR-17-5p in MCF-7 cell was tested respectively with Ep via quantitative RT-PCR. miR-17-5p was to be transected with miR-17-5p mimic and negative control of miR-17-5p mimic (NC). Quantitative RT-PCR, MTT assay, flow cytometry assay, western blot for the proliferation and apoptosis related proteins were performed to determine the function of miR-17-5p in breast cancer cells. The bioinformatic programs TargetScan was used to predict the targets for miR-17-5p. Luciferase reporter gene assay system was used to validate and determine the targets of miR-17-5p. The relation between targets protein levels in breast cancer cells was investigated by western blot.

Results: The expression levels of miR-17-5p was associated with the breast cancer tissues. The levels of miR-17-5p was down-regulated in breast cancer tissues and cells. Ep could inhibit viability of cancer cells in a concentration dependent manner and promote the expression of miR-17-5p in breast cancer cell lines. Over-expression of miR-17-5p induced cell apoptosis and upregulated the expression of p53, p21 and p27. miR-17-5p co-cultured with Ep is better than the other groups. The relative luciferase activity revealed that STAT3 was a potential target gene of miR-17-5p.

Conclusions: Our work will prove that epirubicin regulated the expression of miR-17-5p to strengthen this effect of epirubicin and inhibited the progression of breast cancer.

Introduction

Breast cancer is the most common malignant tumor for women, which has been ranked first in women's cancer-related death (1). Although the incidence of breast cancer in China is lower than that in western countries, the nation-wide average of breast cancer mortality has reached to 26.1 per million. Nowadays the therapeutic options for breast cancer mainly include surgery, radiotherapy, chemotherapy and endocrine therapy (2). Despite comprehensive surgery treatment or chemotherapy, there are still 60–70% of II-III stage patients relapsed in five years (3).

The new class of anticancer anthraquinone epirubicin is known as epirubicin, compared to doxorubicin, which is more efficient and has a broad spectrum (4). It has lower toxicity than doxorubicin and daunorubicin. At present, it is the first choice among same drugs and has been widely used (5). A growing body of evidence proved that epirubicin achieve the goal of cancer therapy by inserting between adjacent base pairs, and the DNA double helix forming a reversible binding, interfering with the process of transcription, inhibiting the synthesis of RNA and DNA (6).

MicroRNAs are a class of non-coding RNA at the length of 22nt, through binding 3'end non-coding region of the target gene messenger RNA, inhibiting translation of target gene RNA or affecting the stability of the target gene RNA, which play the roles as oncogenes or tumor suppressor (7). miR-17-5p is overexpressed in lung cancer. It has been reported that miR-17-5p may have synergistic effects through inhibiting the target genes in the progression of diffuse large B cell lymphoma (DLBCL), suggesting that they may become molecular markers for clinical stages (8). miR-17-5p affected the progression of human ovarian cells by upregulating YES1 and downregulating CHAF1A (9)

In our study, we focused on the effects of miR-17-5p on human breast cancer cell growth activity while co-cultured with epirubicin to explore the relationship between epirubicin and miR-17-5p and to develop novel therapeutic tools for preventing and treating breast cancer.

Materials And Methods

Samples. 30 pairs of breast cancer tissues and adjacent normal tissues were collected from the patients diagnosed with breast cancer at the Affiliated Suzhou Hospital of Nanjing Medical University during August, 2016 to January, 2018. All the patients have no radiotherapy and chemotherapy. All the patients have signed informed consent. The study has been approved by the Ethics Committee of the Affiliated Suzhou Hospital of Nanjing Medical University.

Cell culture. The breast cancer cell line MCF-7 and normal breast cell line MCF-10A were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)-GlutaMax (Thermo Fisher Scientific, MA, USA) containing 10% foetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA) and 1% penicillin/streptomycin mix at 37 °C in a humid atmosphere of 5% CO₂.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNAs were isolated from tissues and cells by using TRIzol® (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The TIANScript RT Kit (TIANGEN Biotech, Beijing, China) was used to reverse the RNA to complementary DNA according to the manufacturer's protocol. The target genes expression was detected using miScript SYBR Green PCR Kit (Qiagen, Germany) on a 7900 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The $2^{-\Delta\Delta Ct}$ method were used to analyse the relative mRNA quantification. Expression data were normalized to the geometric mean of housekeeping gene GAPDH or U6 to control the variability in expression levels.

Cell viability detection assay. Cells were divided into five groups: control group, NC group, mimic group, Ep group, and mimic + Ep group. The cells in logarithmic growth phase were seeded in a 96-well plate containing epirubicin with different concentration. After incubation for 24 h, cells were treated with MTT (Beyotime, Shanghai, China) in fresh medium for 4 h. The dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve the formazan product. subsequently, the optical absorbance was tested at 490 nm using a microplate reader.

Cell transfection. The miR-17-5p mimic and negative control of miR-17-5p mimic (NC) were obtained from Guangzhou Ribobio (Guangzhou, China). The miRNAs were transfected into cells by using Lipofectamine2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

Western blot. RIPA lysis buffer (Beyotime, Shanghai, China) was purchased from Amresco, USA and used to extract the protein from all groups of cells. 10% SDS-PAGE was used to separate the proteins, and then the proteins were transferred onto a PVDF membrane. The protein membranes were blocked in 5.0% non-fat milk at room temperature. After 1 h, incubated with anti-STAT3 antibody (ab68153, 1: 2000, Abcam, MA, USA), anti-p53 antibody (ab131442, 1: 1000, Abcam, MA, USA), anti-p21 antibody (ab109520, 1: 1000, Abcam, MA, USA), anti-p27 antibody (ab32034, 1: 5000, Abcam, MA, USA) or anti- β -actin antibody (ab8227, 1: 2000, Abcam, MA, USA) overnight at 4 °C. After being washed by TBAT, the membranes were incubated with horseradish peroxidase conjugated secondary antibodies (ab6721, 1: 2000, Abcam, MA, USA) at room temperature for 1 h. The blots were visualized using The FluorChem imaging system was used to detect the target proteins on the membrane by using an enhanced chemiluminescence method.

Dual-luciferase reporter assay. The wild type (WT) and mutant type (MUT) 3'-UTR region of STAT3 were inserted into pmiR-RB-Report™ vector (Promega, Madison, WI, USA). The miR-17-5p mimic and wild type (WT) and mutant type (MUT) 3'-UTR region of STAT3 were co-transfected into MCF-7 cells. The dual-luciferase reporter assay system (Promega, Madison, WI, USA) was carried out to analyse the luciferase activity.

Statistical analysis. The above experimental steps were performed in triplicate. Data were expressed as means $\pm$ S.D. The statistical differences between two groups were analyzed with Student-T test. ANOVA was performed to evaluate the differences among multigroups. $P < 0.05$ was regarded as significant.

Results

miR-17-5p is downregulated in breast cancer tissues and breast cancer cells. To elucidate the expression level of miR-17-5p in breast cancer tissues and normal tissues, we initially used qRT-PCR to test miR-17-5p level in 30 malignant breast and non-malignant (normal) tissues, respectively. As shown in Fig. 1A, we found it was down-expressed in breast cancer tissues compared with the normal tissues. Furthermore, we determined the expression of miR-17-5p in breast cancer cell line (MCF-7) and the normal breast cell line (MCF-10A). The expression level of miR-17-5p was significantly lower in t MCF-7 s compared with the non-malignant MCF-10A cell line (Fig. 1B).

Cell viability were inhibited by epirubicin in a dose-dependent manner. MTT assay was applied into detect the inhibitory growth rate of the cells. MCF-7 cells were cultured in vitro. The cells in logarithmic growth phase were seeded in a 96- well plate, cultured for 48 h and treated with different concentrations of epirubicin (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 μ g/ml) to observe cell viability of MCF-7 cells. As depicted in Fig. 2A, with the concentration of epirubicin, the cell viability of MCF-7 cells decreased gradually. Cell viability were inhibited by epirubicin in a dose-dependent manner.

miR-17-5p is upregulated by epirubicin in breast cancer cells. In order to investigate whether a link exists between epirubicin and miR-17-5p expression. We treated the MCF-7 cells with 1 µg/ml epirubicin, after incubation for 24 and 48 h, we determine the expression level of miR-17-5p using qRT-PCR. As expected, the level of miR-17-5p was upregulated, and with the increase of the time of epirubicin, the level of miR-17-5p increased gradually (Fig. 2B). To further confirm the hypothesis, miR-17-5p mimic and negative control of miR-17-5p mimic (NC) were transiently transfected into breast cancer MCF-7 cells. After transfected miR-17-5p mimic, we treated with 1 µg/ml Ep as a experimental group. The expression level of miR-17-5p in the four groups were tested by qRT-PCR. The result confirmed that epirubicin upregulation levels of miR-17-5p in MCF-7 cells (Fig. 2C).

miR-17-5p mimic transfection significantly inhibits proliferation and induces apoptosis of breast cancer cells in vitro. miR-17-5p expression system was established to upregulate the level of miR-17-5p in MCF-7 cells. Cell apoptosis and proliferation on five experimental groups using MTT assay and flow cytometry. As shown in Fig. 3A, compared with NC group, the OD value in mimic group and Ep group was significantly decreased ($p < 0.01$). The OD value of the cells treated with miR-17-5p mimic and epirubicin was significantly lower than that treated with epirubicin alone ($p < 0.05$). After transfected-miR-17-5p and/or cultured with Ep inhibited promoted the apoptosis of the breast cancer cells ($p < 0.01$). The effect of miR-17-5p mimic and epirubicin was more remarkable compared with Ep group ($p < 0.01$).

miR-17-5p directly targets STAT3. The JAK/STAT pathway is pivotal for the transduction of a multitude of signals that are critical for development and homeostasis in mammals (10). Constitutively activated JAKs phosphorylate critical cellular substrates, such as the STAT family, which includes STAT3, that are associated with oncogenic signaling pathways (11). Constitutive activation of STAT3 plays a critical role in cell growth and survival in human solid tumor malignancies and in the up-regulation of the anti-apoptotic genes encoding Mcl-1 and Bcl-xL proteins in human cancer cells (12).

The bioinformatic programs TargetScan was used to predict the targets of miR-17-5p and found that oncogene STAT3 was a potential target gene of miR-17-5p. To further confirm it, luciferase reporter gene assay system was carried out. As shown in Fig. 4A and B, hsa-miR-17-5p regulate the expression of the 3'UTR of luciferase carried STAT3. After mutation of binding site, the regulatory relationship was disappeared. As predicted, STAT3 is a target gene of miR-17-5p.

The mechanisms of combination therapy of epirubicin with miR-17-5p in breast cancer. To further examine whether the conclusions above could be supported by examined the expression levels of STAT3, p53, p21 and p27 using western blot and qRT-PCR. The experimental group cells were divided into five groups and tested the levels of STAT3, p53, p21 and p27 respectively. As shown in Fig. 5A-E, the expression of p53, p21 and p27 in mimic group and Ep group were markedly upregulated, and the expression of STAT3 was down-regulated when compared with NC group. As expected, co-cultured with miR-17-5p mimic and Ep further inhibited the expression of STAT3 and promoted the expression of p53, p21 and p27 compared with Ep group ($p < 0.05$).

Discussion

miRNAs are short (19 to 25-nucleotide) RNAs, processed from hairpin loop structures (pre-miRNAs; 60–110 nucleotides in length) that regulate the expression of protein-coding genes as a result of imperfect complementarity with targeting messenger RNAs (7). The expression levels of microRNAs were valuable for predicting the clinical behavior of cancer. In vertebrates microRNAs have been implicated as regulators of developmental timing, cell proliferation, programmed cell death and fat metabolism, and recently they are found to be abnormally expressed in several types of cancer (13). It is highly likely that the expression levels of individual miRNA molecules play an important role in cancers (14–18). It has been shown that miR-17-5p aberrant expression in many cancers (19–22). So, the purpose of this study is to explore the antagonistic effect and underlying mechanisms of combination therapy of epirubicin with miR-17-5p in breast cancer. Firstly, in the present study, we analysis the expression levels of miR-17-5p in breast cancer tissues and cells, and found that miR-17-5p was lower in breast cancer tissues and cells. So, we hypothesize that miR-17-5p may contribute to cure breast and thus chose miR-17-5p for the further investigation in our study.

Epirubicin, a common chemotherapeutic agent, has been reported has strong killing effect to various cells. In our study, we observe that cultured MCF-7 cells with different concentrations of epirubicin affect differently the growth of the cells. Even though, remarkable advances have been made in anti-cancer mechanisms of epirubicin, it needs more further studies on the relationship of epirubicin and miR-17-5p. Through the study, we demonstrated that Epirubicin could promote the expression of miR-17-5p in breast cancer cell lines and it was in a time-dependent manner. Moreover, our results suggest that after transfected miR-17-5p mimic and cultured with Ep more significantly inhibits proliferation and induces apoptosis of breast cancer cells *in vitro* when compare with other groups.

To elucidate the molecular mechanism by which miR-17-5p inhibits MCF-7 cells proliferation, we identified STAT3 as a direct and functional target of miR-17-5p. STAT3 is a latent cytoplasmic transcription factor, originally discovered as a transducer of signomial from cell surface receptors to the nucleus (23). Compelling evidences suggested that STAT3 was constitutively activated in many cancers and played a pivotal role in tumor growth and metastasis. It regulated cellular proliferation, invasion, migration, and angiogenesis which were critical for cancer metastasis (24). In order to further confirm the molecular mechanism, the western blot and qRT-PCR assay were carried out to test the level of STAT3, p53, p21 and p27 in RNA and protein level. All the functional experiments, we presented evidences for miR-17-5p coordination with Ep to further inhibited the expression of STAT3 and promoted the expression of p53, p21 and p27 as compared with NC group.

Taken together, we confirmed that miR-17-5p was a key mediator in cell apoptosis by targeting STAT3. The expression of miR-17-5p was improved by EP. In addition, after transfected miR-17-5p mimic and cultured with Ep more significantly inhibits proliferation and induces apoptosis of breast cancer cells. Our study provided a framework to understand the antagonistic effect and underlying mechanisms of

combination therapy of epirubicin with miR-17-5p in breast cancer, and developed therapeutic strategies against breast.

Declarations

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of the Affiliated Suzhou Hospital of Nanjing Medical University (No: 2018-183-01).

Consent for publication

All patients in this study agree on the publication of their person's data.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

Lingling Zhao: Contributes to all the experiment

Zhongjian Zhu: Contributes to all the experiment

Jiang Du and Yuanyu Zhao: Contributes to the data collection and analysis

Dandan Fan: Contributes to the design of the study and the writing of the manuscript

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Not applicable.

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Figures

Figure 1

Expression of IL-8 in lung cancer tissues/ cells compared with normal tissues/ cells A: Compared with normal tissues, the expression of miR-17-5p in breast cancer tissues was significantly decreased. **p<0.01. B: The expression of miR-17-5p in MCF-7 was significantly lower than that in MCF-10A. **p<0.01.

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Figure 2

Epirubicin suppressed the cell viability of breast cancer cells in a dose- and time-dependent manner. A: The OD value of breast cancer cells MCF-7 was negatively correlated with concentration level of epirubicin. B: The expression level of miR-17-5p after treated cells with EP for 24 h, 48 h. The expression of miR-17-5p was increased with the epirubicin-treatment time. * $p < 0.05$, ** $p < 0.01$. C: Compared with

control and NC group, the expression of miR-17-5p in mimic group and mimic+Ep group was significantly increased, especially in mimic+Ep group. $**p<0.01$, $\#p<0.05$.

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Figure 3

Combination of miR-17-5p and epirubicin inhibited the cell viability of breast cancer cells. A: Cells treated with miR-17-5p mimic and/or epirubicin suppressed the proliferation of MCF-7. The effect was more distinctive in mimic+Ep group compared with Ep group. $**p<0.01$, $\#p<0.05$. B-C: The apoptosis rate of breast cancer cell in mimic and Ep groups was significantly increased in comparison with NC group. The apoptosis rate of cells treated with mimic+Ep was significantly higher than that treated with Ep. $**p<0.01$, $\#\#\&p<0.01$.

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Figure 4

The result of luciferase reporter A-B: STAT3 is a direct target of miR-17-5p.

[illegible]

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[illegible]

The result of luciferase reporter A-B: STAT3 is a direct target of miR-17-5p.

Position 252-258 of STAT3 3'UTR

5' ...CUUUGAGCAAUCUGGCACUUUU
 |||
hsa-miR-17-5p 3' GAUGGACGUGACAUUCGUGAAAC

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Figure 5

The combination of miR-17-5p and epirubicin inhibited cell proliferation and promoted cell apoptosis of breast cancer via regulating the expression of STAT3, p53, p21 and p27 A: cells treated with miR-17-5p mimic or epirubicin alone significantly downregulated the protein level of STAT3 and upregulated the protein level of p53, p21 and p27, which was more efficient in mimic+Ep group. B-E: The mRNA level of STAT3 in miR-17-5p mimic or epirubicin was significantly decreased compared with NC group, while the mRNA level of p53, p21 and p27 was significantly increased. Moreover, the cell treated with miR-17-5p mimic or epirubicin was more remarkable. ** $p < 0.01$, #& $p < 0.05$.

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