

Original Article

MiR-21 regulates TGFBI expression in breast cancer cells

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Received November 10, 2015; Accepted January 3, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Breast cancer is a common malignant tumor in women that accounts for 10% of the whole body malignant tumors. Epidemiological investigation showed that breast cancer incidence keep increasing in our country, serious influencing on women's physical and mental health. Breast cancer oncogenesis is a complex biological process, and its pathogenesis is still unclear. In recent years, it was found that TGFBI gene was associated with breast cancer onset, and miR-21 highly expressed in breast cancer tissue. Therefore, this study aimed to clarify the regulation relationship between miR-21 and TGFBI in breast cancer, and investigated miR-21 effect in breast cancer pathogenesis. 10 cases of breast cancer patients receiving excision in XXX hospital were enrolled. Para-carcinoma tissues were treated as control. Real time PCR was applied to test miR-21 and TGFBI mRNA levels. Western blot was performed to detect TGFBI protein expression. Dual luciferase reporter assay was used to determine the relationship between miR-21 and TGFBI. Cell proliferation was tested. Compared with para-carcinoma tissue, miR-21 expression elevated 2.85 times in breast cancer tissue. TGFBI mRNA and protein reduced in cancer tissue, as 0.62 and 0.51 times compared with para-carcinoma tissue. Dual luciferase reporter assay showed that miR-21 can bind with 3'-UTR of TGFBI mRNA. MiR-21 overexpression declined TGFBI mRNA and protein expression in breast cancer cells, and promoted cell proliferation. MiR-21 low expression revealed the opposite results. TGFBI gene is one of the target genes of miR-21. MiR-21 affects breast cancer cells proliferation through regulating TGFBI expression.

Keywords: miR-21, TGFBI, breast cancer

Introduction

Breast cancer is a common malignant tumor in women accounting for about 20% of the new tumors [1]. In recent years, breast cancer incidence presents rising trend in our country. The death number caused by breast cancer occupies the second place of women malignant tumor, only second to lung cancer [2, 3]. Advanced breast cancer is easy to metastasis that seriously threatens patient's life [4]. At present, surgical excision is the major method for breast cancer treatment, accompanied by radiotherapy and chemotherapy [5]. Early detection can make the breast cancer has good therapeutic effect, while metastasis may let the treatment become difficult [6]. Thus, in-depth study breast cancer oncogenesis has important significance to improve the early diagnosis and timely treatment of breast can-

cer. Breast cancer tumorigenesis is a complex biological process, and its pathogenesis is still not clear. MicroRNAs (miRNA) are a kind of small noncoding RNAs with high conservation. Gene encoding miRNA can form mature miRNA under the effect of Drosha and Dicer enzymes [7]. Mature miRNA inhibits target gene expression to accomplish its biological effect by specifically binding with mRNA 3'UTR region [8]. Several studies suggested that miRNAs can regulate about 30% gene expression in vivo, and they involved in cell proliferation, differentiation, apoptosis, and canceration biological processes [9]. In recent years, it was revealed that miRNAs affected multiple tumors oncogenesis by regulating target gene expression and mediating intracellular signaling pathway, including osteosarcoma, liver cancer, breast cancer, gastric cancer, and colorectal cancer [10]. Previous study showed miR-21 highly expressed

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Table 1. Primer sequence

Gene	Forward	Reverse
β -actin	GAGGGAAATCGTGCGTGAC	CTGGAAGGTGGACAGTGAG
TGFBI	ATCACCAACAACATCCAGCA	CCGTTACCTTCAAGCATCGT
U6	GCTTCGGCAGCACATATACTAAAT	CGCTTCACGAATTTGCGTGTTCAT
miR-21	UCAACAUCAGUCUAUAAGCUA	CAGUACUUUUGUGUAGUACAA

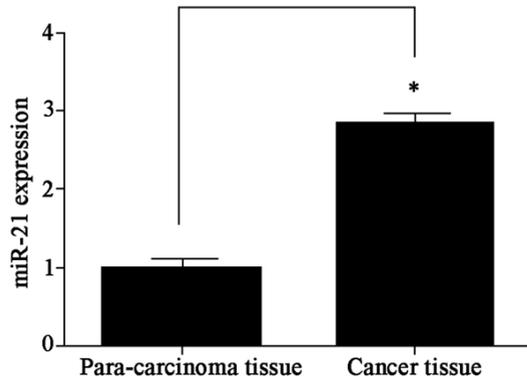


Figure 1. MiR-21 expression in breast cancer tissue and para-carcinoma tissue (* $P < 0.05$).

in breast cancer tissue [11], indicating that miR-21 may participate in breast cancer formation. Based on this, this study explored the mechanism of miR-21 in regulating breast cancer through detecting miR-21 expression in breast cancer tissue and para-carcinoma tissue.

Materials and methods

Main reagents and instruments

Serum-free DMEM medium (Gibco, USA); serum free MEM medium (Gibco, USA); Fetal bovine serum (Gibco, USA); 0.25% trypsin (Gibco, USA); total RNA extraction kit Trizol (Invitrogen, USA); reverse transcription kits (Toyobo Life Science, Japan); SYBR real time PCR kit (Toyobo Life Science, Japan); TBST buffer (Gibco, USA); TGFBI antibody (Abcam, USA); β -actin antibody (Abcam, USA); Real-time PCR amplifier (ABI, USA); CCK8 kit (Dojindo, Japan); Lipofectamine 2000 (Invitrogen, USA); Enzyme-linked immune detector (Bio-tek, USA); Dual luciferase reporter detection system (Promega, USA); Cell incubator (Thermo, USA), etc.

Cell line

Human breast cancer cell line MDA-MB-231 was from Kunming cell bank.

Study objects and specimens

10 cases of breast cancer patients receiving excision between Jan 2012 and Jan 2013 in XXX hospital were enrolled. Tumor tissue and para-carcinoma tissue were collected from the surgery. Para-carcinoma tissue was at least 5 cm away from tumor tissue margin. The samples were stored in liquid nitrogen. Breast cancer tissue was treated as experimental group, while para-carcinoma tissue was considered as control. The study was approved by ethics committee, and all the enrolled patients had signed the informed consent.

Real time PCR

Total RNA was extracted from tissue and cells using Trizol. 1 μ g RNA was incubated at 65°C for 5 min and reverse transcribed to cDNA according to the manual. 1 μ l cDNA was mixed with 1 μ l primers, 10 μ l qPCR Mix, and water to form 20 μ l qPCR reaction solution. Real time PCR was performed on PCR amplifier. The condition was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The primers used were listed in **Table 1**.

Western blot

Tissue or cell was cracked by RIPA (containing protease inhibitor) at 4°C. The tissue was vibrated for 30 s and placed on ice, while the cell was ultra-sonicated for 60 s and centrifuged at 12000 g for 10 min. The supernatant was collected in EP tube and the protein concentration was determined by BSA standard method. 60 μ g protein was separated by 10% SDS-PAGE AND transferred to NC membrane. After blocked in 5% skim milk for 2 h and washed by TBST buffer for three times, the membrane was incubated in primary antibody (1:1000) at 4°C for 8-10 h. After washed by TBST buffer again, the membrane was incubated in secondary antibody (1:10000) at room temperature for 1 h. The protein band was at last exposed to film and analyzed by GIS-2020D gel image analysis system.

Cell transfection

MDA-MB-231 cells in logarithm phase were seeded in 12-well plate at 5×10^4 and incubated for 12 h. The cells were divided into 4 groups,

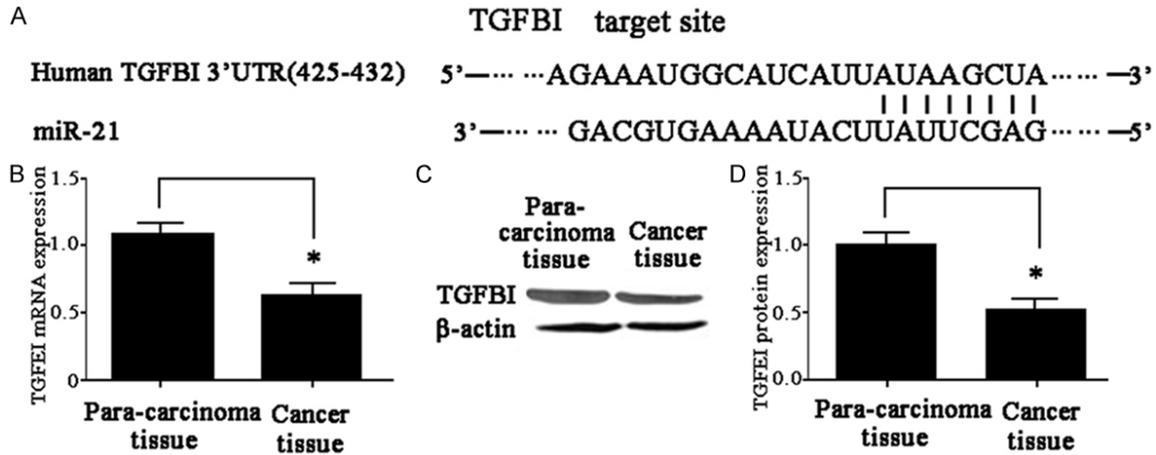


Figure 2. TGFBI expression in breast cancer tissue and para-carcinoma tissue (*P < 0.05). A. miR-21 and TGFBI mRNA 3' UTR binding sequence; B. TGFBI mRNA expression level; C and D. TGFBI protein expression level.

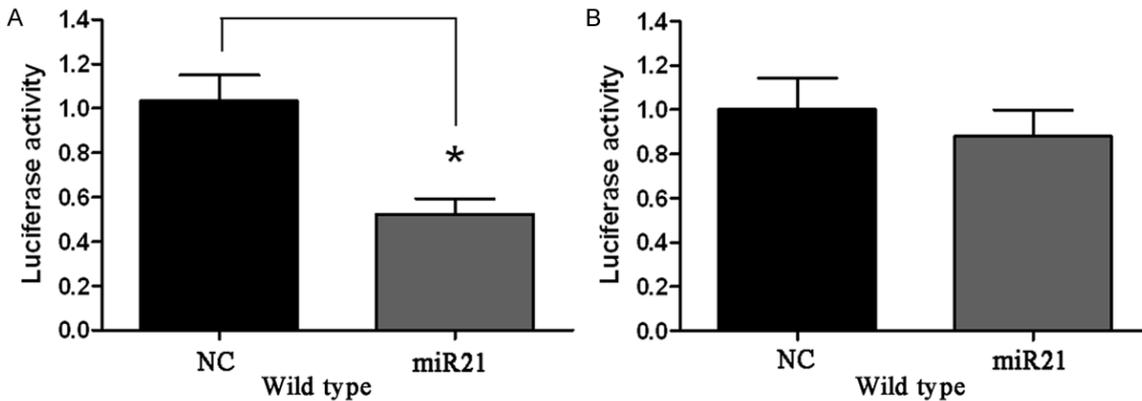


Figure 3. Dual luciferase reporter assay result (*P < 0.05).

including NC group, miR-21 mimic group, NC inhibitor group, and miR-21 inhibitor group. 100 μ l serum free MEM medium was mixed with 3 μ l NC (miR-21 mimic, NC inhibitor, or miR-21 inhibitor) as tube A. Another 100 μ l serum free MEM medium was mixed with 3 μ l lipo2000 as tube B. Tubes A and B were mixed and standing at room temperature for 5 min. 800 μ l mixture was added to each well for 4 h. DMEM complete medium was changed in for 48 h to detect related gene expression.

Dual-luciferase reporter assay

MiR-21 and TGFBI mRNA 3'-UTR sequence were confirmed by Targetscan. Primers were designed based on TGFBI mRNA 3'-UTR. PCR was applied to amplify wild type and mute type segments of TGFBI mRNA 3'-UTR, of which the

mutation site on mute type segment located in the target region of miR-21. After treated by double enzymes restriction and connection, the segment was inserted to pMIR reporter plasmid. After confirmed by PCR amplification, TGFBI wild type and mute type luciferase reporter plasmid was obtained by purification. TGFBI wild type or mute type reporter plasmid, PTK internal reference reporter plasmid, and miR-21 mimic were transfected to MDA-MB-231 cells. After 48 h cultivation, the cells were tested on dual-luciferase reporter detection system (Promega).

CCK8 assay

1×10^4 MDA-MB-231 cells in logarithm phase were seeded in 96-well plate. The cells were divided into 4 groups, including NC group, miR-

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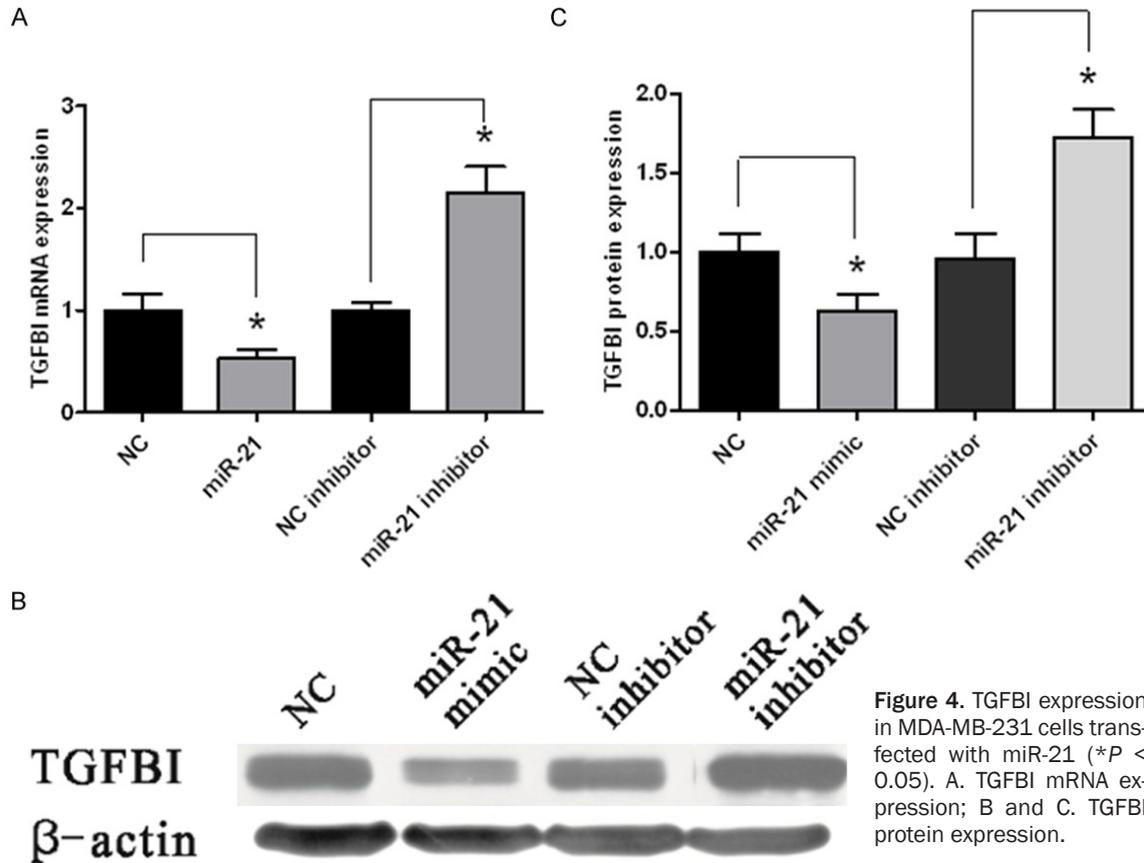


Figure 4. TGFBI expression in MDA-MB-231 cells transfected with miR-21 (* $P < 0.05$). A. TGFBI mRNA expression; B and C. TGFBI protein expression.

21 mimic group, NC inhibitor group, and miR-21 inhibitor group. Each group had six repeated wells. The cells were incubated for 24, 36, 48, 60, 72, 84, and 96 h, respectively. After washed by PBS, the cells were treated with 100 μ l 10% CCK8 solution at 37°C for 4 h. Absorption value was read on enzyme-linked immune detector to draw the growth curve.

Statistical analysis

SPSS 16.0 was used for statistical analysis. The results were presented as mean \pm standard deviation. T test or ANOVA were applied for comparison. $P < 0.05$ was considered as statistical significance.

Results

miR-21 expression in breast cancer tissue

Previous study showed that miR-21 overexpressed in breast cancer tissue. To further explore the role of miR-21 in breast cancer oncogenesis, we tested miR-21 expression in breast cancer tissue and para-carcinoma tis-

sue. Compared with para-carcinoma tissue, miR-21 expression elevated 2.85 times in breast cancer tissue (Figure 1).

TGFBI gene expression in breast cancer tissue

TGFBI was a target gene of miR-21 based on miRanda and Targetscan prediction (Figure 2A). For previous research reported that TGFBI gene was associated with cell proliferation, we tested TGFBI gene expression in breast cancer tissue and para-carcinoma tissue. TGFBI mRNA and protein reduced in cancer tissue, as 0.62 and 0.51 times compared with para-carcinoma tissue (Figure 2B-D).

TGFBI gene is a target gene mediated by miR-21

Dual luciferase reporter assay showed that luciferase activity was obviously inhibited in cells transfected with miR-21 mimic and TGFBI mRNA 3' UTR wild type plasmid, while its activity presented no significant changes in cells transfected with miR-21 mimic and TGFBI mRNA 3' UTR mute type plasmid (Figure 3).

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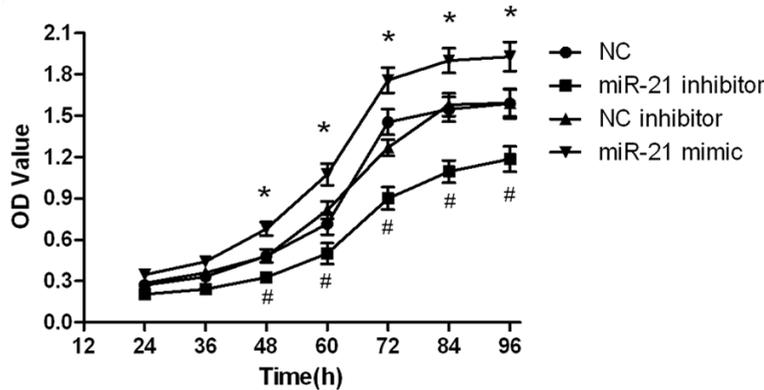


Figure 5. MiR-21 impact on breast cancer cell proliferation. * $P < 0.05$, compared between NC and miR-21 mimic group; # $P < 0.05$, compared between NC inhibitor and miR-21 inhibitor group.

MiR-21 regulates TGFBI gene expression

To further investigate miR-21 regulation on TGFBI gene, we changed miR-21 expression level in MDA-MB-231 breast cancer cells. We transfected NC, miR-21, Inhibitor NC, and miR-21 inhibitor to MDA-MB-231 cells, and detected TGFBI expression after 48 h (**Figure 4**). Compared with NC control, TGFBI expression declined in cells transfected with miR-21 mimic to 0.54 times (mRNA) and 0.63 times (protein), respectively. Compared with NC inhibitor, TGFBI expression elevated in cells transfected with miR-21 inhibitor to 2.15 times (mRNA) and 1.72 times (protein), respectively.

MiR-21 impact on MDA-MB-231 breast cancer cell proliferation

MDA-MB-231 cells were divided into 4 groups, including NC group, miR-21 mimic group, NC inhibitor group, and miR-21 inhibitor group. Each group had six repeated wells. The cells were incubated for 24, 36, 48, 60, 72, 84, and 96 h, respectively. CCK8 was applied to determine cell proliferation (**Figure 5**). Compared with NC group, miR-21 mimic transfection obviously enhanced cell proliferative ability; compared with NC inhibitor group, miR-21 inhibitor transfection significantly weakened cell proliferative ability.

Discussion

Breast cancer is a common female malignant tumor. There are about 1.6 million new cases of breast cancer every year, accounting for 20% of

the female cancer incidence [12]. Europe and the United States are the high incidence area of breast cancer, while China also presents increasing trend [13]. Surgery supplemented by radiotherapy and chemotherapy is the major method for breast cancer treatment, but there is still a large number of patients appear recurrence or metastasis, which seriously impair life and health [14]. Gene therapy in recent years brought new hope for breast cancer early diagnosis and treatment. MiRNAs are a class of

highly conserved noncoding RNAs that regulate target gene expression by complete or incomplete complementary binding with target gene mRNA 3'UTR [15]. It was reported that miRNA was an important regulator in the cell that involved in multiple biological processes including cell proliferation and canceration [16]. Cellular oncogenesis a complex biological process often associated with abnormal cell proliferation [17]. Previous study found miR-21 overexpressed in breast cancer tissue. Our results also confirmed that miR-21 level in breast cancer tissue was obviously higher than that in para-carcinoma tissue. To further investigate the role of miR-21 in breast cancer oncogenesis, we explored miR-21 impact on breast cancer cell proliferative ability and possible mechanism.

Targetscan prediction of miRNA target genes showed that TGFBI and SATB1 were the potential target genes of miR-21. TGFBI protein, a type of secretion protein induced by growth factor β beta secreted protein, is closely related to tumorigenesis [18]. Previous research reported that TGFBI deletion may cause cell excessive proliferation, while TGFBI low expression can promote cancer [19]. Our results revealed that compared with para-carcinoma tissue, TGFBI mRNA and protein overexpressed in breast cancer tissue. Previous study confirmed that miR-21 low expressed in breast cancer tissue. To test the relationship between miR-21 and TGFBI gene, we constructed TGFBI mRNA 3'UTR wild type and mute type reporter plasmids, and co-transfected the reporter plasmids

and miR-21 mimic to breast cancer cells. We further applied dual luciferase reporter system to detect the fluorescent signal. The results demonstrated that miR-21 can obviously decline the fluorescent signal of transfected TGFBI wild type plasmid, suggesting that TGFBI was the target gene of miR-21. Next, we tested TGFBI expression after changing miR-21 level in breast cancer cells and found that promoting (or inhibiting) miR-21 expression lead to TGFBI downregulation (elevation), indicating that miR-21 regulates TGFBI expression in breast cell cancer. Further investigation showed that miR-21 overexpression obviously enhanced breast cancer cell proliferation, while miR-21 low expression weakened cell proliferation, suggesting that miR-21 can affect breast cancer cell proliferation.

In conclusion, TGFBI is the target gene of miR-21. MiR-21 affects breast cancer cell proliferation through regulating TGFBI expression. However, breast cancer oncogenesis is a complex biological process affected by multiple factors [20]. Further investigation is needed to explore the mechanism of breast cancer tumorigenesis.

Disclosure of conflict of interest

None.

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