Roles of miR-106b and sox4 in breast cancer

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Abstract: Objective: This study aims to investigate the expression, biological function and associated mechanisms of miR-106b in the breast cancer. Methods: We collected 53 fresh tumor samples from patients with breast cancer to detect miR-106b expression by qRT-PCR method. Through transfection of miR-106b mimics into MCF-7 cells, the effects on the proliferation, cell cycle, invasion and metastasis were checked by CCK-8 assay, Transwell migration assay, and Flow cytometry methods, respectively. The potential target gene sex determining region Y box 4 (SOX4) was detected in MCF-7 cells after over-expressing miR-106b. The dual luciferase assay was used to validate that SOX4 was directly targeted by miR-106b through complementary binding to 3'UTR of mRNA sequence. Results: MiR-106b expression was significantly decreased in tumor tissues of breast cancer, and the expression level was negatively correlated with the clinical staging. MiR-106b expression was lower in tumor tissues with lymph node metastasis than in tumor without lymph node metastasis. Results in vitro showed that miR-106b inhibited the proliferation, invasion and metastasis of MCF breast cancer cells. SOX4 protein expression was down-regulated after over-expression of miR-106b. The results from dual luciferase assay further validated that miR-106b directly targeted SOX4 to regulate its expression. Conclusion: MiR-106b expression was significantly down-regulated in breast cancer tumor cells, which was also associated with lymph node metastasis and clinical stages. Through directly down-regulating SOX4 expression, miR-106b plays as tumor suppressor gene to inhibit the proliferation, invasion and metastasis of MCF cells.

Keywords: MiR-106b, sox4, breast cancer

Introduction

Breast cancer is a malignant tumor occurred in breast ductal epithelial cells, which is a common cancer in women and accounts for about 7%-10% of all malignancies [1, 2]. According to the statistics report released by the American Cancer Society in 2013, breast cancer ranks the first among the morbidities of all types of female cancers, and the mortality is up to 14%, which is a great threat to women’s health [3]. In recent years, with the improvement of clinical diagnosis and treatment, the 5-year survival rate for breast cancer is increasing, but the metastasis is still one of the important causes of poor prognosis in breast cancer patients [4]. The metastasis in breast cancer is difficult for clinical treatment, and the molecular mechanisms are still unclear.

Numerous studies demonstrate that miRNA widely participates in the development of breast cancer as one kind of post-transcriptional factor [5, 6]. MicroRNA (miRNA) is one class of highly conserved non-coding RNA with length about 18-22 nucleotides. Through complementary pairing with the 3’UTR of target mRNA, miRNA can inhibit translation of target genes [7]. Studies showed that expression profiles of miRNA changed significantly in breast cancer, and miRNA played roles of oncogenes or tumor suppressor genes in the proliferation, invasion and metastasis of breast cancer cells [8, 9]. MiR-125 and miR-145 were reported to inhibit metastasis of breast cancer tumor cells through regulating the expression of erb-b2 receptor tyrosine kinase 2 (ERBB2) and ADP-ribosylation factor 6 (ARF6) respectively [10, 11]. MiR-106b is discovered in recent years and located on...
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chromosome 7, which belongs to a member of miR-106b-25 cluster. MiR-106b is highly homologous with oncogene miR17-92 cluster [12]. It was reported that miR-106 was highly related to the metastasis of cancer cells [13, 14], although the regulatory roles in breast cancer are still unclear. In this study, we investigated the expression and associated mechanisms of miR-106b in breast cancer by qRT-PCR, Western blot and other molecular technology. Our findings may provide experimental basis to further illustrate the function of miRNAs in breast cancer.

Materials and methods

Sample collection

This study collected 53 breast cancer patients who received surgical resection and 16 cases of normal breast tissues in Nanjing Medical University Affiliated Jiangsu Cancer hospital from May 2013 to March 2014. All the cancer tissues were confirmed by pathological diagnosis after surgery, and all specimens were invasive ductal cancerous tissues with thickness more than 3 mm and quality more than 30 mg. The average age for all patients was 44.6 ± 1.5 years old from 25 to 86 years old. Among 53 patients, 31 cases had lymph node metastasis while other 22 cases had no metastasis. Based on the staging criteria of breast cancer by the American Joint Committee on Cancer (AJCC) 2003, all samples were consisted into 15 cases of stage I, 27 cases of stage II, and 11 cases of stage III. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Nanjing Medical University.

MCF-7 cell culture

The breast cancer cell line MCF-7 cells were purchased from cell bank of pathological science, CAS. After rapidly thaw from liquid nitrogen, MCF cells were cultured with 10 ml DMEM medium in 37°C within 5% CO₂ incubator for 24 h. Then the medium was replaced with 5 ml DMEM medium containing 10% FBS. The cells were cultured in incubator and the medium was replaced once two days. When spread to about 90%, the cells were used to for passage based on routine procedure.

RNA extraction from breast cancer tissues

After frozen by liquid nitrogen, the tissues were ground into powder. Total RNA was isolated from tissues using 1 ml Trizol (Invitrogen, California, US) reagent per 100 mg, according to the manufacture’s protocol. Phenol-chloroform method was used to extract total RNA. The cDNA was reverse transcribed from total RNA by polyA tailing method with Reverse Transcription System (Takara, Dalian, China) and stored in -20°C. The reverse transcription system included: 5 μL miRNA template, 2× miRNA Reaction Buffer Mix 10 μL, 0.1% BSA 2 μL, miRNA PrimeScript RT Enzyme Mixture (Takara, Dalian, China) 2 μL, and H₂O 1 μL. The reaction was performed at 37°C for 60 min with PolyA primer.

Detection of miR-106b by qRT-PCR

The miR-106b was detected by qRT-PCR in cancer and normal control tissues. The U6 was used as internal reference. The primers for miR-106b were 5’-ACATTCAATTGCTGTCGGT-3’ and Uni-miR qPCR Primer provided in kit (KAPA BIOSYSTEMS, Boston, USA). The system was included: 5 μL cDNA, 10 μl Mix, 0.5 μl PCR Forward Primer, 0.5 μl Uni-miR qPCR Primer, and 13 μL ddH₂O, total 30 μL. The cycle conditions were the following: 95°C for 10 min, and followed by 40 cycles at 95°C for 30 s, 60°C for 30 s. Each sample had 3 replicates. The relative expression of miR-106b was calculated by the 2ΔΔT method.

miR-106b mimics transfection into MCF-7 cells

The MCF-7 cells were cultured by antibiotics free DMEM medium containing 10% FBS, and divided into 3 groups based on transfection: miR-106b mimics group, Negative control group, and control group (Blank). When cell density reached about 70%~90%, 20 pmol/L miR-106b mimics (1.25 l) and lipo2000 (Invitrogen, California, USA) (1 l) was added into EP tubes containing 50 l Opti Memi medium respectively. The 2 tubes were mixed together after 5 min incubation, and then followed by 20 min incubation at room temperature. The mixture was added into culture plates and DMEM medium containing 10% FBS was replaced after 6 h. The changes of biological characters were detected by CCK-8 assay, cell cycle and Transwell assay. The cells were harvested after transfection 48 h and Western blot was used to detect the SOX4 protein expression.

Proliferation changes by CCK-8 assay

The cells in Negative control group and transfection group were seeded into 96-well plates
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(2×10^3) respectively, and each group had 3 replicates. The CCK-8 (Beyotime Biotechnology, Beijing, China) solution was added to each well. At 24 h, 48 h, and 72 h, the absorbance of each well at 490 nm wavelength were measured, and the proliferation curves of MCF-7 cells were generated.

Transwell migration assay and invasion assay

The migration assays were performed using the Transwell chamber (Corning Company, New York, USA). The MCF-7 cells were seeded into the upper chambers (1×10^5 cell/well) that were incubated by 200 µl of serum free DMEM medium, while the bottom of the chamber was incubated with 600 µl DMEM medium containing 10% fetal bovine serum. After culture for 24 h, cells were fixed by formaldehyde, washed by PBS, and stained by Giemsa staining for 1 min. After washing 3 times, pictures of the cells were taken under a microscope with 5 random views. The number of invaded cells was counted to estimate the capabilities of cell migration.

To evaluate the changes of cell invasion, Matrigel (BD, New Jersey, USA) was used to simulate the extracellular matrix environment. Matrigel was thawed at 4°C overnight from -20°C before experiment. After dilution by serum free DMEM medium by 1:2 proportion, 50 µl was smeared into upper chamber and solidified into gel at 37°C for 60 min. All procedures were performed on ice and all tips were pre-cooled at 4°C. After culturing 72 h, the procedure for cell invasion was the same as that of cell migration.

Cell cycle by flow cytometry

After transfection 24 h, 1×10^6 cells in each group were washed by pre-cooled PBS twice. Then Cell Cycle Assay Kit (BD, New Jersey, USA) was used to detect the cell cycle based on the protocol. Briefly, total 200 l A solution was added to incubate at room temperature for 10 min, and then 150 l solution liquid was added to incubate at room temperature for 10 min. Finally, 120 l C solution was incubated for 10 min in dark. Then flow cytometry was used and results were analyzed by Modfit software.
Western blot analysis

MCF-7 cells in each group were washed by precooled PBS for 2 times, and then were lysed with RIPA lysis buffer and PMSF on ice for 5 min. The supernatant was centrifuged at 12000 g/min for 10 min at 4°C. The supernatant was reserved. Before electrophoresis, the proteins was mixed with equal volume of 2×SDS loading buffer and boiled for 10 min. Then 5 μL was loaded into SDS-PAGE (100 V constant voltage) and then transferred to PVDF membrane (300 mA constant current at 4°C for 2 h). The primary antibody was mouse anti-human sox4 (1:1000) and rabbit anti-human GAPDH antibody (1:5000). The second antibodies were HRP-conjugated goat anti-rabbit IgG (1:1000) and goat anti-mouse IgG (1:5000). All the antibodies were purchased from Abcam Company (Boston, US). Finally, the membrane was developed by enhanced chemiluminescence plus reagent.

Dual luciferase assay

Based on the bioinformatics prediction, the wild-type 3’UTR and the mutant 3’UTR of SOX4 were synthesized in vitro and were cloned into the downstream of pMIR-REPORT luciferase vector by Spe-1 and HindIII enzyme. HEK293T cells were co-transfected with miR-106b mimics and wild-type SOX4 3’UTR or the mutant 3’UTR. After transfection for 24 h, cells were lysed and luciferase intensity was measured by GloMax 20/20 luminometer (Promega, Wisconsin, USA) based on the standard protocol of the luciferase kit (Promega, Wisconsin, USA). The intensity of Renilla was used as control, and the fluorescence intensity in different groups was analyzed.

Statistical analysis

The SPSS 16.0 software was used to do statistical analysis. All the data were shown as the mean ± SD, and difference were determined by two-tailed Student’s t-test. P<0.05 was considered as statistically significant.

Results

MiR-106b expression in different samples of breast cancer

To detect miR-106b expression among different samples, qRT-PCR was performed. Com-
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Compared with normal tissues, miR-106b expression (0.37±0.06) was significantly down-regulated in patients with breast cancer (P<0.05), shown in Figure 1A. Among the different pathological stages, miR-106b expression was decreased significantly when stage was increased (P<0.05), as shown in Figure 1B. MiR-106b expression was also significantly lower in patients with lymph node metastasis than in patients without metastasis (P<0.05, Figure 1C). The results indicated that miR-106 was down-regulated in breast cancer tumor samples, and it was closely related to the metastasis and clinical staging in breast cancer.

Influence of miR-106b on proliferation, invasion and migration of MCF-7 cells

To study the effects of miR-106b on proliferation of MCF-7 cells, CCK-8 assay was applied to detect the changes of proliferation after overexpression of miR-106b. Based on the CCK-8 assay, it was shown that proliferation rate of MCF-7 cells was lower in miR-106 mimics group than in normal control group and negative control group (as shown in Figure 2A). The results indicated that over-expressed miR-106b inhibited the proliferation of MCF-7 cells, and the lower expression of miR-106b in breast cancer tissues may promoted the proliferation, invasion and metastasis.

To investigate the roles of miR-106b in the invasion and migration of MCF-7 cell lines, we applied Matrigel invasion chambers and Transwell method to check the changes of invasion and migration on differently transfected cells. Compared with negative control group (48.5±5.42), the number of trans-member cells...
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was significantly decreased in miR-106b mimics group (27.5±4.30) (P<0.05). The invasion assay also indicated that the number of cells through transwell was significantly decreased in miR-106b mimics group (16.7±1.2) than in negative control group (29.5±2.3, P<0.05). The details were shown in Figure 2B and 2C. The results indicated that miR-106b inhibited the invasion and migration of MCF-7 cells.

Effects of miR-106b on cell cycles of MCF-7 cells

To check the influence of miR-106b on cell cycles, we applied flow cytometry to detect the changes of MCF-7 cell cycle. As Figure 3 shown, compared with Negative control group, MCF-7 cells were appeared in G1/S phase arrest in miR-106b mimics group, which indicated that increased miR-106b can inhibit the proliferation of MCF-7 cells through regulate G1/S phase transition.

SOX4 protein expression by Western blot

To study whether miR-106b can influence SOX4 protein expression in MCF-7, Western blot was used to detect the SOX4 expression in different group. As Figure 4 shown, compared with the negative control group, SOX4 protein expression was significantly down-regulated in MCF-7 cells with over-expressed miR-106b (P<0.05). The results indicated that miR-106b might play roles in breast cancer through regulating SOX4 expression.

Sox4 is directly targeted by miR-106b

To determine whether SOX4 was directly targeted by miR-106b, we detected the GFP intensity by dual luciferase assay after co-transfection. As shown in Figure 5, fluorescence of cell lysis was significantly down-regulated in cells co-transfected with miR-106b mimics and pMIR-REPORT-wild type plasmid than in negative control group (P<0.05), while there was no significant difference between group co-transfected with miR-106b mimics and pMIR-REPORT-mutant and negative control group (P>0.05). The results from dual luciferase assay indicated that miR-106b could regulate SOX4 expression through complementary binding to 3’-UTR of SOX4 mRNA.

Discussion

Breast cancer is a one of common tumors that seriously threatens women’s health and the main clinical treatment is surgery, chemotherapy and radiation therapy, but the 5-year survival rate is still not ideal [15, 16]. Early metastasis
in breast cancer is the main reason for poor prognosis [17]. Similar with other tumors, the occurrence and development of breast cancer is a complex process that involves multi-gene, multi-stage, and multi-factor. Previous studies focused more on the identification of oncogenes and tumor suppressor genes [18]. In recent year, miRNA becomes a hot topic as it can directly regulate oncogenes and tumor suppressor genes. Bioinformatics prediction shows that one miRNA can regulate hundreds of target genes, and tumor development is usually caused by the expression disorders of a large number of genes. So miRNA may play important roles in tumor. Studies showed that varieties of miRNAs were close related to breast cancer. Up-regulation of miR-10b can promote invasion and migration in breast cancer [19]. Let-7 can help breast cancer cells to remain the properties of cancer stem cells [20]. MiR-125 family is related to the prognosis of patients with breast cancer through regulating Her2 expression [21]. All these indicated that miRNA played important roles in the development and progression of breast cancer.

MiR-106b belongs to miR-17 family that abnormally expressed in many kinds of tumors and participated to the proliferation, invasion and metastasis of tumor cells [22]. In this study, we demonstrated that miR-106b expression was significantly decreased (P<0.05), and it was significantly down-regulated in lymph node metastasis group (P<0.05). MiR-106b expression was decreased gradually with clinical staging. All these indicated that miRNA played important roles in the development and progression of breast cancer. After over-expression of miR-106b in MCF-7 cells, the proliferation, invasion and migration were inhibited, which indicated that miR-106b inhibited proliferation, invasion, and migration of breast cancer cells. In addition, miR-106b can inhibit cell proliferation through G1/S phase arrest. The results further validated that lower expression of miR-106b in tumor tissues of breast cancer may promote the metastasis. It is important to identify the target genes of miRNA to declare the roles of miRNA. Bioinformatics prediction shows that SOX4 is one of potential target of miR-106b. SOX4 is a transcription factor that can promote invasion and metastasis of varieties of tumors [23, 24]. The results from Western blot showed that sox4 expression was reduced after miR-106b over-expression, which indicated that miR-106b might regulate SOX4 expression. For further validate this, we applied dual luciferase assay. The results further indicated that miR-106b regulated sox4 expression through complementary binding to 3'UTR of SOX4 mRNA sequence.

In summary, miR-106b can inhibit the proliferation, invasion and metastasis of breast cancer through directly down-regulating sox4 protein expression. MiR-106b mainly plays as tumor suppressor roles in breast cancer, which has great clinical value as it may become a potential treatment target.

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Disclosure of conflict of interest

None.

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References

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