**Original Article**

**Targeted regulation of miR-15b on EZH2 in breast carcinoma and its effect on cell biological function**

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**Abstract:** Objective: To explore the role of miR-15b on EZH2 in breast carcinoma and its biological function. Methods: The breast carcinoma tissues and adjacent tissues of 85 patients with breast carcinoma were collected. miR-15b and EZH2 in the tissues were tested by qPCR, and the correlation of miR-15b and EZH2 with the clinicopathological features of patients was analyzed. Breast carcinoma MCF-7 cell line was also used, miR-15b and EZH2 were measured by qPCR, the transfection of the cells was carried out, the changes of cell proliferation ability, invasion and migration after transfection were analyzed, the cell cycle was detected, and the targeted relationship between miR-15b and EZH2 was verified. Results: miR-15b level was low in breast carcinoma, and EZH2 level was high in breast carcinoma. miR-15b and EZH1 levels in breast carcinoma tissue had a correlation with tumor size, lymph node metastasis and distant metastasis. Transfection of miR-15b mimic and si-EZH2 evidently reduced the proliferation of MCF-7 cells, the colony forming efficiency, and the invasion and migration of cells. The ratio of G0/G1 cells increased evidently, and miR-15b had a targeted relationship with EZH2. Conclusion: miR-15b is low-expressed and EZH2 is high-expressed in breast carcinoma tissues. miR-15b targeting EZH2 can reduce the proliferation, invasion, and migration of breast carcinoma cells.

**Keywords:** miR-15b, EZH2, breast carcinoma, proliferation ability, invasion and migration, targeted regulation

**Introduction**

Breast carcinoma is a malignant tumor type in females, and it is also the main reason for carcinoma death in females [1]. According to statistics, there were about 2.1 million new cases of breast carcinoma in the world in 2018, accounting for nearly a quarter of carcinoma cases among women [2]. Breast carcinoma is a highly heterogeneous disease, which can be divided into different molecular subtypes according to the content of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [3]. Its pathogenesis is complex, and its clinical characteristics and treatment responses are quite different. Although many disordered molecular pathways have been found in breast carcinoma, the progression of therapeutic methods is limited by its extensive transcriptional regulation and genomic heterogeneity [4]. With the improvement of modern medicine, the 5-year survival of breast carcinoma in situ is 98%, but metastatic breast carcinoma is still incurable due to the lack of reliable therapeutic targets [5].

MicroRNAs are highly conserved non-coding small RNA molecules with 18-25 nucleotides, which act in post-transcriptional adjustment of genes [6]. Studies have confirmed that microRNAs are closely related to tumor formation, malignant progression and prognosis, and have the potential of being important therapeutic targets [7]. miR-15b belongs to the miR-15 family [8], which can regulate apoptosis induced by the B-lymphoma-2 (Bcl-2) gene in various cells [9]. Studies have shown that miR-15b was reduced in breast carcinoma cells, and miR-15b was reduced in carcinoma tissues and enhanced in adjacent tissues in tumor tissues and corresponding adjacent tissues [10]. EZH2 (Enhancer of zeste homolog 2), located on chromosome 7q35, is the core catalytic protein of
Effect of miR-15b targeting EZH2 in breast carcinoma

comb repressor 2 (PRC2), which catalyzes the trimethylation of the 27th lysine of histone H3 (H3K27) and mediates the gene silencing of the target gene [11]. As an important biomarker in invasive breast carcinoma, EZH2 can play a role in regulating the progression of malignant tumors derived from tissues, such as breast carcinoma and prostate carcinoma [12]. However, there are few reports on the interaction between miR-15b and EZH2. This study mainly explored the relationship of miR-15b and EZH2 in breast carcinoma and their effects on the biological function of breast carcinoma cells.

Materials and methods

Research participants

The tissue specimens were selected from 85 women with breast carcinoma, the tissues were surgically removed in The First Affiliated Hospital of Anhui Medical University from October 2018 to October 2019. All patients were female with an average age of (46.2±11.6) years. Inclusion criteria: All patients were diagnosed with breast carcinoma by pathological diagnosis, and all patients had pathological specimens of breast carcinoma tissues and adjacent tissues; none of the patients had received chemotherapy, radiotherapy and biological immunotherapy before pathological sampling; the patient’s clinical medical records were complete. This study was approved by the ethics committee of our hospital, and patients gave their signed informed consent. Exclusion criteria: patients who had received chemotherapy, radiotherapy and biological immunotherapy before pathological sampling; patients who were unwilling to participate in this study.

Cell culture

MCF-7 was obtained from ATCC. The cell strain was cultivated in RPMI 1640 medium including 10% FBS at 37°C with 5% CO₂ and with saturated humidity. After 2-3 passages, the experiment was carried out in the logarithmic phase of growth.

qPCR

Trizol extraction kit (Invitrogen, USA) was applied to extract total RNA from tissue cells. miR-15b, EZH2 and GAPDH primers were synthesized by Sangon Biotech. miR-15b, F: 5'-TGAATTGACTTGACCATAATAGA-3', R: 5'-AATGTTGCTGTATCCCT-3'; EZH2, F: 5'-AATTGACATGCACGTCTAGA-3', R: 5'-GCTGTATCC-TTCGCTGTTCC-3'; GAPDH, F: 5'-GGAGCGAGATCCCTCCAAAAT-3', R: 5'-GGCTGTTGTCATACTTCTCATGG-3'. Then, RNA was reverse transcribed into cDNA by PrimeScript RT kit. Using cDNA as template, fluorescence quantitative detection PCR was performed by 2× TaqMan rapid universal PCR reaction solution at 95°C for 3 min. The reaction conditions were as follows: 95°C for 3 min; 95°C for 20 s; 68°C for 30 s, for a total of 30 cycles; maintained temperature at 72°C for 10 min. U6 was applied as internal reference for miR-15b, and GAPDH for EZH2. All reactions were set with three multiple wells, and quantitative testing was performed using 2⁻ΔΔCt method.

Cell treatment and transfection

MCF-7 cells in a logarithmic growth phase were cultivated into 6-well plates and divided into the control group, miR-15b over-expression group, blank over-expression group, EZH2 interference group, and blank interference group. The miR-15b over-expression group and blank over-expression group were transfected with miR-15b mimic and miR-15b mimic blank control (mimic NC), respectively. The EZH2 interference group and blank interference group were transfected with si-EZH2 and si-EZH2 blank control (si-NC), respectively. After 24 h of cultivation, follow-up tests were performed.

Cell proliferation

MTT assay was applied. The transfected cells in each group were made into cell suspension, and then the cells were counted and cultivated in a 96-well plate (1×10⁴ cells/well). After 6, 12, 24 and 48 hours of culture, respectively, 20 μL MTT was added at 20 μL/well. After 4 hours, the medium was discarded and 200 μL DMSO was added and shaken at low speed for 10 min. The absorbance at 490 nm was tested by microplate reader, which represents the cell proliferation ability. The experiment was repeated three times.

Colony forming efficiency

Transfected cells of each group were inoculated into a 6-well plate (2×10² cells/well), cultivated in an incubator until the cells were visible...
Effect of miR-15b targeting EZH2 in breast carcinoma

to the naked eye, washed with PBS 3 times, fixed with 4% paraformaldehyde for 0.5 h, then given 500 μL Giemsa staining solution to stain for 30 min, and rinsed with running water to remove the staining solution. The number of cell clones was counted under the microscope (more than 50 cells were counted as one clone), and the colony forming efficiency was tested. Colony forming efficiency = (number of clones/number of inoculated cells) ×100%.

Transwell invasion test

A volume of 50 μL of diluted Matrigel matrix glue was spread in the upper chamber of Transwell, and placed in an incubator at 37°C for 30 min. The transfected cells of each group were selected, and the cells were re-selected in serum-free medium, and 700 μl RPMI 1640 culture medium containing 20% serum was put into each well (lower chamber) of 24-well plate. The chambers were placed in the wells, and about 5000 cells were added to each chamber, and the volume was increased to 200 μl with serum-free medium. After 48 h of culture, the medium in the chamber was discarded, and the cells that did not migrate in the upper chamber membrane were removed with cotton swabs and chambers were rinsed with PBS three times. The cells were then Fixed with 4% paraformaldehyde for 20 min, washed with PBS, and dyed with 0.1% crystal violet for 20-25 min. The remaining crystal violet was rinsed off with running water and cells were air dried. Pictures in different visual fields were taken under an inverted microscope, and the cells were counted. The migration of cells was detected by the same method. Except that the upper chamber of the Transwell was not coated with Matrigel matrix glue, the other steps were the same as the invasion experiment. The experiment was repeated 3 times and the average value was taken.

Cell cycle detection

Trypsin was applied to digest the transfected cells in each group, the cells were then collected in a centrifuge tube, centrifuged, washed once with 4°C pre-cooled PBS, then fixed with 1 mL of 70% ethanol pre-cooled at 4°C, cultivated overnight at 4°C, centrifuged again and washed once with 4°C pre-cooled PBS. PI staining solution was prepared and 0.5 mL of staining solution was put onto cells. The cells were slowly resuspended, and bathed in the dark at 37°C water for 45 min, and then checked.

Double luciferase reporter gene

MCF-7 cells in a logarithmic growth phase were digested with pancreatin to prepare the cell suspension, inoculated in a 6-well plate (2×10^5 cells per well), and cultivated in an incubator at 37°C for 24 h with 2 mL complete medium per well with 5% CO₂ to make the cell fusion degree reach 70-80%. EZH2-WT 3'-UTR and EZH2-MUT 3'-UTR vectors were established and co-transfected with miR-15b mimics and NC mimics according to the instructions of X-treme gene HP transfection reagent (Roche). The luciferase activity of each group was detected referring double luciferase reporter gene detection kit (Promega).

Statistical analysis

The data were tested by SPSS 21.0. Measurement data were represented by mean ± SD, single factor analysis of variance was applied for data comparison among multiple groups, t test was applied for pair-wise comparison. The counting data were represented by n (%) and compared by chi-square test. The difference was statistically significant with P<0.05. GraphPad Prism 8.0 was applied for data illustrations.

Results

Relationship of miR-15b and EZH2 with clinicopathological features in breast carcinoma tissues

Using the average content of miR-15b and EZH2 as the middle value, an expression level higher than the average value was taken as a high expression, and an expression level lower than the average value was taken as the low expression. The results revealed that the expression of miR-15b and EZH2 in breast carcinoma were not correlated with the age, menopause, ER expression, PR expression, HER2 expression and TNM stage (P<0.05). The expression of miR-15b and EZH2 was correlated with tumor size, lymph node metastasis and distant metastasis (P<0.05) (Table 1).

miR-15b and EZH2 in breast carcinoma

miR-15b was reduced and EZH2 was increased in 85 samples of breast carcinoma tissue and
adjacent tissues, and the difference was statistically significant (P<0.001) (Figure 1).

**miR-15b and EZH2 in breast carcinoma cells of each group**

Compared with the Blank group, miR-15b in MCF-7 cells transfected with miR-15b mimic was evidently increased, while EZH2 was evidently reduced (P<0.05). Compared with the si-NC group, miR-15b in MCF-7 cells transfected with si-EZH2 had no obvious difference in values (P<0.05), but EZH2 was evidently reduced (P<0.05) (Figure 2).

**Proliferation and invasion of breast carcinoma cells in each group**

MCF-7 cells were transfected with miR-15b mimic and si-EZH2 respectively, and the cell proliferation and colony forming efficiency were evidently reduced (P<0.05) (Figure 3A, 3B). The invasion and migration of MCF-7 cells in miR-15b mimic group and si-EZH2 group was higher than that in Blank group and si-NC groups (Figure 3C, 3D).

**Verification of cell cycle and targeted correlation in breast carcinoma**

MCF-7 cells were transfected with miR-15b mimic and si-EZH2, and the ratio of G0/G1 cells increased evidently, which blocked the cell cycle of breast carcinoma in G0/G1 phase (Figure 4A). Double luciferase reporter gene was applied to determine that the fluorescence activity reduced when EZH2-WT was co-transfected with miR-15b mimics (P<0.01), while the fluorescence activity of the other co-transfect-
Effect of miR-15b targeting EZH2 in breast carcinoma

Figure 1. Expression of miR-15b and EZH2 in breast carcinoma. A: Expression of miR-15b in different tissues; B: Expression of EZH2 in different tissues; *** indicates compared with the adjacent tissues, P<0.001.

Figure 2. Expression of miR-15b and EZH2 in breast carcinoma cells of each group. A: Expression of miR-15b in breast carcinoma cells of each group; B: Expression of EZH2 in breast carcinoma cells of each group; *** indicates compared with Blank group, P<0.001; ** indicates compared with Blank group or si-NC group, P<0.01.

Discussion

The pathogenesis of breast carcinoma has not been found, and its occurrence and development are complicated with many factors, multi-steps and multi-stages. Therefore, it is of great significance to find out the biological indicators in the occurrence and development of breast carcinoma in order to carry out targeted individualized treatment for breast carcinoma patients. microRNAs have sequence conservation, strict spatiotemporal profiles and tissue specificity [13]. mRNA plays a key regulatory role in different physiological processes of cells, such as proliferation, apoptosis, migration, invasion, differentiation and metabolism. According to the different functions of target genes downstream of microRNAs, microRNAs can play the role of an oncogene promoter and a tumor suppressor in the process of tumor formation and malignant progression [14]. At present, a variety of abnormally expressed microRNAs have been found in breast carcinoma. microRNAs have the potential to be effective targets for tumor therapy. EZH2 is highly expressed in many solid tumors including breast carcinoma, and EZH2 level is negatively correlated with the prognosis of patients. In addition, EZH2 can promote the proliferation of breast tumor starter cells [15].

In this study, miR-15b and EZH2 in breast carcinoma tissues and their effects on the biological function of breast carcinoma cells were explored. The results revealed that miR-15b level was low in breast carcinoma tissues and EZH2 level was high in breast carcinoma tissues. The content of miR-15b and EZH2 in breast carcinoma had a correlation with tumor size, lymph node metastasis and distant metastasis. Some specific microRNAs are related to specific biological types of breast cancer, for example, miR-30 is related to ER and PR status. In ER and PR negative tumors, the expression of miR-30 is observed to be down-regulated. miR-213 and miR-203 are related to tumor grade, and miR-213 and miR-203 are highly expressed in tumors with high tumor grade [16]. miR-206 targets the 3’-untranslated region of ER protein, and the concentration of miR-206 is negatively correlated with ER protein [17, 18]. In this study, there are similar results. After transfection of miR-15b mimic and si-EZH2, the proliferation and colony forming efficiency of MCF-7 cells reduced evidently, suggesting that the proliferation of MCF-7 cells...
Effect of miR-15b targeting EZH2 in breast carcinoma

was hindered and the number of invading and migrating cells reduced evidently. The ratio of G0/G1 cells increased evidently, suggesting that transfection of miR-15b mimic or si-EZH2 could block the cell cycle of breast carcinoma in G0/G1 phase, thus inhibiting cell proliferation. miR-15b regulates the progression of breast cancer by targeting genes. It is reported that miR-15b regulates the invasion and migration of breast cancer by targeting MTSS1 [19], and Kratassiouk et al. [20] reported that miR-15b can regulate the cell cycle of breast cancer cells by targeting CPEB1. On the basis of the above research, our study showed that miR-15b could influence the invasion and migration of breast cancer cells by regulating EZH2. According to the double fluorescein reporter gene experiment, miR-15b could target and regulate the expression of EZH2.

In carcinoma cells, obvious epigenetic changes can promote the formation of a malignant phenotype [21]. Epigenetic mechanisms such as DNA methylation and chemical modification of histone tails act in transcriptional regulation. Histone modification plays a key role in chromatin formation and transcriptional regulation [22]. DNA is closely arranged around histones to form nucleosomes. The N-terminal tail of the nucleosome extends out of the nucleosome core and forms a large number of dot like protein modifications, such as acetylation, methylation, phosphorylation, ubiquitination or ADP ribosylation. EZH2 gene is the core catalytic protein of comb repressor 2, which catalyzes the trimethylation of the 27th lysine of histone H3 and mediates the gene silencing of the target gene. EZH2 is a proto-oncogene with a wide range of biphasic transcriptional regulators, whose regulatory function is mainly to silence tumor suppressor genes by the methyltransferases. These tumor suppressor genes are mainly involved in tumorigenesis and transcriptional activation of carcinoma end-stage genes [23,
The results revealed that the expression disorder may be an important initiating factor in the process of tumor occurrence and development, and inactivation may be an effective treatment for many carcinomas. Studies have shown that increased protein expression is evidently related to triple negative breast carcinoma and low survival rate [25]. With people's cognition and understanding of miRNAs, the explorations of miRNAs in breast carcinoma are developing. It has been found that many miRNAs are abnormally expressed in breast carcinoma, such as miR-133a [26], miR-320 [27], and miR-34a [28]. At the same time, many miRNAs were found to participate in the mediation of EZH2 expression, such as miR-26a [29] and miR-138 [30].

The shortcomings of this study are as follows: the sample size is too small to provide sufficient and powerful data analysis to prove the influence of miR-15b and EZH2 on breast cancer, and further mechanistic research is needed when the results of cell experiment are applied to clinic. In conclusion, miR-15b can regulate EZH2 in breast carcinoma, and over-expression of miR-15b can inhibit the proliferation, invasion and migration of breast carcinoma cells. They play an important role in the progression of breast carcinoma, and are hoped to become important biological targets and prognosis evaluation indexes of breast carcinoma.

Disclosure of conflict of interest

None.

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Effect of miR-15b targeting EZH2 in breast carcinoma


