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MiR-216b-5p and HDAC8 Inhibit Breast Cancer Cell Proliferation, Metastasis and Invasion

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ABSTRACT

The objective of this study was to investigate the mechanism of MiR-216b-5p and HDAC8 inhibiting the proliferation, metastasis and invasion of breast cancer cells. Tissue samples (28) were collected, and malignant and normal tissues were confirmed pathologically. The breast cancer cell line MDAMB-231 and the immortalized normal breast epithelial cell line MCF-10A were used in this study. Western blot analysis of miR-216b-5p and HDAC8 protein expression in tissue samples; reverse transcription quantitative PCR (RT-qPCR) real-time analysis of miR-216b-5p, HDAC8 mRNA expression in cell lines; MTT detection of cell proliferation; wound healing test and transwell assay were used to evaluate the ability of breast cancer cells to metastasize and invade; colony formation test was conducted to detect the effect of HDAC8 on the cells. We found that the expression of miR-216b-5p protein in breast cancer group was lower than that in healthy control group (P<0.05); the expression of HDAC8 protein in the group was higher than that in healthy control group (P<0.05). The MDA-MB-231 group had lower miR-216b-5p mRNA expression than the MCF-10A group (P<0.05), and the MDA-MB-231 group had higher HDAC8 mRNA expression than the MCF-10A group (P<0.05). At 24th h after cell line culture The cell proliferation of miR-216b-5p inhibitor group was higher than that of miR-216b-5p mimic group and control group (P<0.05). At 48th h after cell line culture, the cell proliferation of miR-216b-5p mimic group was lower than that of control group and miR-216b-5p inhibitor group (P<0.05). The cell proliferation of control group was lower than that of the miR-216b-5p inhibitor group (P<0.05). At 72th h after cell line culture, the miR-216b-5p mimic group had lower cell proliferation than miR-216b-5p inhibitor group (P<0.01). And the control group had higher cell proliferation than miR-216b-5p mimic group (P<0.05). The miR-216b-5p inhibitor group had more cell metastasis and invasion than the miR-216b-5p mimic group and the control group (P<0.05). The miR-216b-5p mimic group had less cell metastasis and invasion than the miR-216b-5p inhibitor group and the control group (P<0.05). Compared with the control group and the empty vector group, the HDAC8 gene knockout group had less colony formation (P<0.05). Compared with the miR-216b-5p mimic group, the expression of HDAC8 protein and HDAC8 mRNA increased in the miR-216b-5p inhibitor group (P<0.05). The miR-216b-5p mimic group had lower HDAC8 protein expression and HDAC8 mRNA expression than the control group(P<0.05). It is concluded that HDAC8 is an oncogene that can promote the proliferation and development of breast cancer. MiR-216b-5p has antitumor effect on HDAC8 and can inhibit the proliferation and development of breast cancer.

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QZ and YY selected the research samples and carried out cell test. MY and JC compared the experimental results. MT made further medical analysis. All authors conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript.

Key words
MiR-216b-5p, HDAC8, Breast cancer, Cell metastasis, Cell proliferation

INTRODUCTION

Breast cancer is the most common malignant tumor among women all over the world, accounting for 26% of all cancers in new cases every year. Histone deacetylase 8 (HDAC8) is a newly identified HDAC, which is related to many diseases, especially hematological malignancies (Li *et al*., 2017b). Recent studies have shown that the higher expression of HDAC8 may be related to tumor progression, pathogenesis and prognosis (Wang and Yu, 2018). Recently, some studies have shown that the regulation of Hippo-YAP signal by HDAC8 may promote the metastasis of TNBC cells, thus introducing HDAC8 as a potential target for TNBC treatment (Yuan *et al*., 2017a).

MiR is called a small non-coding RNA, which has a length of 19-25 bp and can be cut from 70-100 bp hairpin pre-miRNA precursor (Liu *et al*., 2019). Single-stranded mature miR binds to the 3' untranslated region (3'UTR) of target mRNA, and regulates the block of translation or degradation of target mRNA molecules (Li *et al*., 2018). According to the literature, many abnormal expressions of miR are related to various human malignant tumors. Among them, miR-216b-5p is a newly recognized miR, which can be used as a tumor suppressor (Shen *et al*., 2019). HDAC8 is a potential target of inhibition related to MicroRNA (miRs) (Wang and Yu, 2018). Bioinformatics analysis shows that miR-21 may target HDAC8, which

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leads to the decrease of carcinogenicity of its protein (Hao *et al*., 2017). In addition, miR-21 is considered as an anti-tumor micro-RNA for many types of malignant tumors (Xu *et al*., 2017). Recent studies have shown that the expression of miR-216b-5p is decreased in the breast cancer cells line (Li *et al*., 2017a). It is also found that the anti-tumor effect of miR-216b-5p in breast cancer cells is exerted by directly targeting cyclin E1 mRNA (Sun *et al*., 2017). With regard to nasopharyngeal carcinoma, researchers have proposed that the antitumor effect of miR-216 can be achieved by PKC and KRAS (Vancurova *et al*., 2018). The association of miR-216 and HDAC8 with breast cancer progression is still unknown. In addition, as far as we know, no other studies can evaluate the role of miR-216 and HDAC8 in carcinogenesis of breast cancer. Therefore, in this study, we aimed to study the levels of miR-216b-5p and HDAC8 in cancerous tissues obtained from breast cancer patients and adjacent non-cancerous tissues, with the specific goal of evaluating the potential role of HDAC8 and miR-216 in human breast cancer.

MATERIALS AND METHODS

Medical ethics issues

The experiment obtained the written informed consent of all subjects. In this case, the hospital's ethics committee approved the research, and the animal experiment scheme was approved by the animal research ethics committee.

Tissue samples and cells

From April 2018 to June 2019, 28 tissue samples from breast cancer patients were collected, which were pathologically confirmed malignant and normal tissues. The malignant tumor tissue samples were named as breast cancer group, and the nearby healthy tissues were healthy control group. The breast cancer cell line MDAMB-231 and the immortalized normal mammary epithelial cell line MCF-10A were used for this study. They were purchased from the International Center for Genetic Engineering and Biotechnology (ICGEB). After collecting cells, the cell lines were inoculated into RPMI-1640 medium supplemented with 10% FBS.

Cell transfection

MDA-MB-231 cell line was cultured in a 6-well plate at a density of 1.5×10^5 cells/well, and grew to 90% fusion. Cells were transiently transfected with 100 nM miR-216b-5p simulation, negative control and inhibitor (Exiqon). Next, we transfected the cells with Lipofectamine 2000 according to the manufacturer's instructions. After transfection, the cells were stored in the medium containing 10%FBS for up to 72 h. Finally, we extracted the total RNA and protein and used them for further study.

CRISPR/Cas9 mediated knockout of HDAC8 gene

The expression of HDAC8 in MDA-MB-231 cell line was knocked out by KO strategy based on CRISPR/Cas9. CRISPR sgRNA was designed by crisper.mit.edu, and pCAG-eCas9-GFPU6-gRNA vector (Addgene 79145) without ITR element was digested with BbsI enzyme to clone gRNA. The prepared eCas9/gRNA expression vector was co-transfected into the cell line using Lipofectamine 2000 (Intel Logan Company, USA).

Colony formation test

MDA-MB-231 cell line was transfected in 6-well culture dish by miR-216b-5p simulation, negative control, inhibitor and HDAC8 knockout vector. In the medium supplemented with 20% FBS, the plate was covered with 0.6% agar. A total of 1,000 cells were prepared in 0.3% agar and incubated at 37°C and 5% CO_2 for 14 days. The resulting colonies were stained with 0.04% crystal violet for 40 min and washed with PBS again. Finally, the number of colonies per well was counted.

Experimental grouping

According to the experimental requirements, the cultured cells were divided into the following groups: MCF-10A group (cultured normal mammary epithelial cell line), MDA-MB-231 group (breast cancer cells line), miR-216b-5p mimetic group (mir-216b-5p overexpression in MDA-MB-231 cell line), mir-216b-5p inhibitor group (low expression of miR-216b-5p in MDA-MB-231 cell line) and HDAC8 gene knockout group (low expression of HDAC8 in MDA-MB-231 cell line).

Western blot

All samples were dissolved in RIPA buffer containing a mixture of protease and phosphatase inhibitor, and incubated at 4°C for 10 min. The protein concentration was measured using BCA protein detection kit (Bio basic, Canada). After electrophoresis of 40μg lysate in 12%SDSPAGE, the gel was transferred to PVDF membrane (Millipore Company, USA). The membrane was sealed with skim milk powder (5%) and incubated with primary antibody overnight. On the second day, all samples were incubated with HRP labeled secondary antibody (MN 55413). At last, ECL detection kit (Sigma-Aldrich) was used to detect the signal, and Image J 1.34I software (NIH) was used to scan and analyze the membrane.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA containing small RNA was extracted from cancerous tissues, normal tissues and cell lines by RNX™-

Plus reagent (Cinnagen Company, Iran). According to the user manual, cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Ki. The real-time quantitative PCR was carried out using SYBR Premix ExTaqII kit (Takara Company, Japan) and Rotor-Gene 6000 instrument (Corbett Research company, Australia). According to the manufacturer's instructions, the expression of MiRs was quantified using SYBRR Premix ExTaqTM kit (Takara Company, Japan). HPRT and SNORD47 (U47) were used to standardize mRNA and miR data, respectively.

Determination of cell proliferation

MTT assay was used to evaluate the proliferation ability of cells. 48 h after transfection, the cells were inoculated in 96-well plate at the rate of 1×10^3 cells/well. 20 µl MTT solution (0.5 mg/ml) were added to each well at 24h, 48h and 72h after transfection. Cells were incubated at 37°C for 4h, and 150 µl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was read at 570 nm using a multifunctional enzyme-linked immunosorbent assay microplate reader (SpectraMax M2 Company, USA). The experiment was repeated for at least 3 times.

Metastasis and intrusion detection

Wound healing test was used to evaluate the metastasis ability of breast cancer cells. Transfected cells were seeded and cultured in a 6-well plate ($5x10^5$ cells per well). When the cell density was close to 90%, a 200µl plastic pipette was used to scrape the fused cell monolayer on a straight line. Prompted, and washed with sterile PBS. The cells were further cultured in a medium containing 1% FBS for 48 h. Scratch closure was photographed at 12h, 24h and 36h under an optical microscope (Olympus IX50, Tokyo, Japan). Image-Pro Plus software (version 5.1, Media Cybernetics Company, USA) was used to calculate the cell metastasis ability of each group.

Invasion was determined in a 24-well plate precoated with Matrigel. After transfection, MCF-7 or MDA-MB-231 cells (at the rate of 5×10^3 cells/well in 100µl serum-free medium) were inoculated into the upper chamber, and 600µl medium containing 10% FBS was placed in a Petri dish. Cells in the lower chamber were incubated at 37°C for 24h. Cells in the upper chamber were removed with cotton swabs. The invaded cells were fixed with methanol for 15 min, stained with 0.1% crystal violet, and scored by counting with inverted microscope using at least five random fields of view. The experiment was repeated for at least 3 times.

Colony formation test

At first, MCF-7 and MDA-MB-231 cell lines were transfected with miR-216b-5p, negative control, inhibitor and HDAC8 knockout vector in 6-well culture dish. In the medium supplemented with 20% FBS, the plate was covered with 0.6% agar. A total of 1,000 cells were prepared in 0.3% agar and incubated at 37°C and 5% CO_2 for 14 days. The resulting colonies were stained with 0.04% crystal violet for 40 min and washed with PBS again. Finally, the number of colonies per well was counted.

Statistical analysis

All the data in this study were processed by SPSS20.0 statistical analysis software (IBM Company, USA). The measurement data was expressed by "mean ±standard deviation" ($\bar{x}\pm s$). The comparison between groups was made by one-way analysis of variance or repeated measures analysis of variance, and the pairwise comparison between groups was made by LSD-t test; the counting data was expressed by percentage (%), and the comparison between groups was analyzed by χ^2; $P<0.05$ indicated statistically significant difference.

RESULTS

Protrin expression of MiR-216b-5p and HDAC8

Western blot analysis shows that the breast cancer lines had lower miR-216b-5p protein expression than the healthy control group ($P<0.05$), and higher HDAC8 protein expression than the healthy control group (Table I).

Table I. miR-216b-5p and HDAC8 protein expression (±s) in healthy control (MCF-10A) and breast cancer cell line (MDA-MB-231) determined by Western blot analysis.

Groups	n	miR-216b-5p	HDAC8
MCF-10 A cells	20	1.87±0.23	0.86±0.05
MDA-MB-231 cells	20	1.16±0.14	1.72±0.19
t value		5.068	6.594
P value		0.017	0.028

mRNA expression of miR-216b-5p and HDAC8

The mRNA expression of miR-216b-5p and HDAC8 in cell lines was analyzed by PCR in real time. The MDA-MB-231 group had lower miR-216b-5p mRNA expression than the MCF-10A group ($P<0.05$), and had higher HDAC8 mRNA expression than the MCF-10A group ($P<0.05$) (Table II).

MiR-216b-5p overexpression inhibits MDA-MB-231 cell proliferation

The MTT assay showed that the cell proliferation of

miR-216b-5P inhibitor group was higher than that of miR-216b-5p mimic group and control group at 24h after cell line culture ($P<0.05$). On the 48thh, the cell proliferation of miR-216b-5p mimic group was lower than the control group and mir-216b-5pinhitor group ($P<0.05$), while that of control group decreased compared with mir-216b-5p inhibitor group ($P<0.05$). On the 72thh, the cell proliferation of miR-216b-5p mimic group was lower than that of miR-216b-5p inhibitor group ($P<0.01$), while that of control group increased compared with miR-216b-5p mimic group (P<0.05) (Table III)

Table II. miR-216b-5p and HDAC8 mRNA expression (±s) in healthy control (MCF-10A) and breast cancer cell line (MDA-MB-231) determined by Real-time PCR analysis.

Groups	n	miR-216b-5p	HDAC8
MCF-10A cells	20	2.13±0.38	1.03±0.18
MDA-MB-231 cells	20	1.25±0.24	1.85±0.35
F value		5.169	6.394
P value		0.011	0.005

Table III. Effect of overexpression of miR-216b-5p on proliferation of MDA-MB-231 cell line as determine by MTT assay (%, Mean±S).

Groups	n	24h (%)	48h (%)	72h (%)
Control	20	13.24±2.51	16.82±3.06	21.69±3.01
MiR-216b-5p mimic	20	10.26±2.06	12.64±2.57	13.57±2.85
MiR-216b-5pin-hibitor	20	17.39±3.18	24.57±3.69	36.75±4.52
F value		11.265	10.734	9.127
P value		0.026	0.034	0.001

Effect of miR-216b-5p on metastasis and invasion

The number of cell metastasis and invasion of mir-216b-5p inhibitor group was higher than that of miR-216b-5p mimic group and control group ($P<0.05$). Compared with mir-216b-5p inhibitor group and control group, the number of cell metastasis and invasion of miR-216b-5p mimic group decreased ($P<0.05$). Overexpression of miR-216b-5p could inhibit metastasis and invasion of MDA-MB-231 cells (Table IV).

HDAC8 participates in cell colony formation regulated by miR-216b-5p

To determine whether HDAC8 is involved in the progression of breast cancer inhibited by miR-216b-5p, HDAC8 was knocked out in MDA-MB-231 cell line by CRISPR/Cas9 method. On the 5th day, there was no difference in cell colony formation between HDAC8 knockout group and control group and empty vector group ($P>0.05$). On the 15th day, HDAC8 knockout group had less cell colony formation than control group and empty vector group (Table V).

Table IV. Effect of miR-216b-5p on metastasis and invasion of miR-216b-5p mimic and inhibitor cells.

Groups	n	Cell metastasis (one)	Cell invasion (one)
control	20	169.66±31.07	176.84±45.29
MiR-216b-5 pmimic	20	134.57±26.12	122.35±21.79
MAiR-216b-5 pinhibitor	20	315.26±74.69	234.15±52.87
F value		31.626	49.238
P value		0.001	0.001

Table V. Effect of miR-216b-5p on cell colony formation (±s).

Groups	n	5th day	15th day
Control	20	23.62±5.33	62.71±15.25
Empty vector	20	21.49±5.18	54.16±14.82
HDAC8 gene knockout	20	17.59±4.68	26.47±6.35
F value		10.628	31.569
P value		0.218	0.001

MiR-216b-5p direct targeting of HDAC8

MiR-216b-5pinhibitor group had higher HDAC8 protein expression and HDAC8 mRNA expression than miR-216b-5p mimic group ($P<0.05$), and miR-216b-5p mimic group had lower HDAC8 protein expression and HDAC8 mRNA expression than control group ($P<0.05$) (Table VI).

Table VI. miR-216b-5p direct targeting (±s).

Groups	n	HDAC8protein expression	HDAC8 mRNA expression
Control	20	1.36±0.22	1.58±0.12
miR-216b-5 pmimic	20	0.86±0.12	0.95±0.05
miR-216b-5 pinhibitor	20	2.13±0.35	2.25±0.41
F value		15.322	19.238
P value		0.001	0.001

DISCUSSION

In this study, the effects HDAC8 and miR-216b-5p on breast cancer progression were evaluated. Here, we showed that miR-216b-5p was down-regulated in human breast cancer tissues and breast cancer cells. We also observed that miR-216b-5p was negatively correlated with HDAC8 level. Our results indicated that miR-216b-5p down-regulated HDAC8 expression by directly targeting HDAC8-3β-UTR. We proved that after miR-216b-5p was overexpressed, HDAC8 decreased significantly. Drug resistance to chemotherapy drugs and invasion and metastasis of breast cancer cells are the main causes of breast cancer-related death. Studies have shown that the changes of histone protein are directly related to the occurrence of breast cancer. Previous studies have shown that HDAC8 is highly overexpressed in triple-negative breast cancer, and the up-regulation of HDAC8 is the reason for the late stage, especially in triple-negative breast cancer and the poor prognosis and poor treatment response of breast cancer (Su and Hu, 2017). Therefore, we can prevent the development of tumor by inhibiting this protein as a potential therapeutic target. Recently, more and more attention are attached to the targeted inhibition of HDAC8 as a potential cancer cell growth inhibitor in vivo and *in vitro* (Xie *et al*., 2017). Consistent with previous studies, the current findings indicate that HDAC8 is overexpressed in clinical breast cancer specimens and breast cancer cells. We confirmed the carcinogenicity of HDAC8 in breast cancer by functional loss experiment. We found that the carcinogenic function of HDAC8 was decreased after transfecting the breast cancer cells with HDAC8-KO-vector. It has also been reported that the expression changes of different miRNA are the factors affecting the pathogenesis of breast cancer. Previous studies have revealed the antitumor effects of miR-216 in nasopharyngeal carcinoma, colorectal cancer, breast cancer and hepatocellular carcinoma (Guo *et al*., 2017). MiR-216 has also been proposed as a potential regulator of apoptosis by interacting with autophagy (Wang *et al*., 2019). Although all studies have emphasized the anti-tumor properties of miR-216b-5p, different cancer research methods have introduced various potential targets for miR-216b-5p, such as UDP-glucuronyl transferase in hepatoma cell lines, translation-controlled tumor protein and pyridol 5'-phosphate oxidase in pancreatic cancer cells during the development of invasive ductal carcinoma of human breast (Yuan *et al*., 2017b). MiR-216b is a tumor suppressor and plays an important role in human cancer progression (Kong and Qiu, 2018). The expression disorder of miR-216b is found in cancer, which may affect the proliferation and invasion of cancer cells through the interaction with other genes (Zhang *et al*., 2019). However, the role of miR-216b-5p in HDAC8 regulation has not been studied. Our results explained that miR-216b-5p could inhibit the proliferation, cell growth and colony formation of breast cancer by regulating the expression of HDAC8. Due to various histopathological characteristics of breast cancer subtypes, the molecular mechanisms involved in disease development and progression are very diverse. β-catenin/Wnt signaling pathway is an important molecular mechanism to initiate epithelial-mesenchymal transition. This mechanism also involves the tumorigenesis and metastasis of breast cancer. Therefore, EMT targeted therapy has created broad prospects for the treatment of breast cancer. In addition, it has been previously proved that HDAC8 may activate Wnt /β-catenin signaling pathway (Zhou *et al*., 2019). The proposed mechanism of cancer inhibition by microRNA may involve targeting genes that affect EMT process, such as HDAC8. The anti-tumor properties of miR-216b-5p may be applied in this way (Chao *et al*., 2020).

To sum up, our results revealed that miR-216b-5p had anti-tumor effect on HDAC8 and could inhibit the proliferation and development of breast cancer. Our data indicated that the inhibition of HDAC8 by miR-261-5p might be proposed as a potential treatment for future research (Mou *et al*., 2019).

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Statement of conflict of interest

The authors have declared no conflict of interest.

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