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The effect of miR-138 on the proliferation and apoptosis of breast cancer cells through the NF-κB/VEGF signaling pathway

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ABSTRACT

The analyze the effect of miR-138 on the proliferation and apoptosis of breast cancer cells through the NF-κB/VEGF signaling pathway is the Objective of this experiment. For this aim, the endometrial stem breast cancer cell line MCF-7 was cultured in vitro, and the overexpression mimic miR-138 mimics and the inhibitor anti-miR-138 were transfected into the endometrial stem breast cancer cell line MCF-7, which was set to overexpress miR-138 group and interfere with miR-138, and set up negative control of overexpression and negative control of inhibitor. Observe the cell proliferation and apoptosis ability of each group, and the changes in tumor necrosis factor-α (TNF-α), interleukin 1β, 6, 18 (IL-1β, IL-6, IL-18) levels, and compare the Bax of each group, NF-κB, VEGF protein expression level. Results showed that the proliferation ability of the miR-138 overexpression group was significantly lower than that of the miR-138 overexpression control group (P<0.05); the proliferation ability of the miR-138 interference group was significantly higher than that of the miR-138 interference control group (P<0.05). The apoptosis rate, caspase-3 and caspase-9 expression levels of the miR-138 overexpression group were significantly higher than those of the miR-138 overexpression control group (P<0.05); the apoptosis rate, caspase-3 and caspase-9 expression levels of the miR-138 interference group were significantly lower than those of the miR-138 interference control group (P<0.05). The expression levels of IL-1 β, IL-6, IL-18 and TNF - α in the miR-138 overexpression group were significantly lower than those in the miR-138 overexpression control group (P < 0.05). The protein expression levels of Bax, NF-κB and VEGF in the miR-138 overexpression group were significantly lower than those in the miR-138 overexpression control group (P < 0.05); the protein expression levels of Bax, NF-κB and VEGF in the miR-138 interference group were significantly higher than those in the miR-138 interference control group (P <0.05). The proliferation ability of the miR-138 overexpression group was significantly lower than that of the miR-138 overexpression control group (P < 0.05); the proliferation ability of the miR-138 + NF-κB overexpression group was significantly higher than that of the miR-138 overexpression group (P<0.05). The apoptosis rate of the miR-138 + NF-κB overexpression group was significantly lower than that of the miR-138 overexpression group (P < 0.05). Then MiR-138 can significantly inhibit the proliferation of breast cancer cells, promote apoptosis, and regulate the expression of inflammatory factors in the cells. It is speculated that the related mechanism may be related to the negative regulation of the NF-κB/VEGF signaling pathway.

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Introduction

Breast cancer is a common female malignant tumor in the clinic, which is caused by the uncontrolled proliferation of mammary epithelial cells under the action of various carcinogenic factors. The early major symptoms of breast cancer manifest the nipple discharge, breast lump and the enlargement of lymph nodes in the axilla, seriously affecting women's health and quality of life (1). In the terminal cancer of the breast cancer, the distant metastasis of cancer cells may occur, resulting in lesions of multiple organs and seriously threatening patients' lives. The pathogenesis of breast cancer has not been completely clear yet. Related data show that the occurrence and development of breast cancer is an uncontrolled process of complex molecular regulation, which is closely related to the cell-matrix adhesion, abnormal signal transduction and inactivation of oncogenes and tumor suppressor genes (2). Therefore, an in-depth understanding of the underlying molecular mechanism

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of breast cancer is of great significance for finding effective targets to block tumor formation. miRNAs, as a type of small non-coding RNA, play an important regulatory role in the cell adhesion, proliferation and angiogenesis by targeting the regulation of one or more RNAs and inhibiting the expression of genes that are target RNAs for translation and interpretation, which is closely related to the pathogenesis of breast cancer (3). The nuclear factor kappa B (NF-κB) is an important nuclear transcription factor, and its activity is closely related to various biological processes, which is widely involved in the gene regulation in multiple pathological processes, such as the inflammation, immunity and cell proliferation (4). Vascular endothelial growth factor (VEGF) can be involved in the formation of blood vessels during the ectopic transplantation of endometrial cells, playing an important role in the formation process of breast cancer (5). This research aims to analyze the effect of miR-138 on the proliferation and apoptosis of breast cancer cells through the NF-κB/VEGF signaling pathway.

Materials and Methods

Experiment reagents and instruments

Endometrial stem breast cancer cell line MCF-7 (Shanghai Aulu Biological Technology Co., Ltd.) MTT solution (Shanghai Fantai Biotechnology Co., Ltd.) DAPI solution (Beijing Baiaolaibo Technology Co., Ltd.) ELISA kit (Shanghai Guduo Biotechnology Co., Ltd.) Trizol pyrolysis liquid (Guangzhou Dongsheng Biotech Co., Ltd.) Protein loading marker (Qingdao Haosail Science CO., Ltd.) Bax, NF-κB, VEGF antibody (Wuhan Yipu Biotechnology Co., Ltd.)

Inverted microscope (Beijing Jiayuan Xingye Technology Co., Ltd.) Pipette (Shanghai Jizhi Biochemical Technology Co., Ltd.) PCR Amplifier (Nanjing Vedeng Medical Co., Ltd.) Water bath kettle (Jiangsu Genloci Biotechnologies Inc.) Protein electrophoresis apparatus (Beijing Popular Science Ganke Technology Development Co., Ltd.) Protein transfer membrane instrument (Saibai'ao (Beijing) Technology Co., Ltd.) Desk centrifuge (Shanghai Fuze Trading Co., Ltd.) Autoclave (Nanjing Vedeng Medical Co., Ltd.)

Cell culture and grouping

The endometrial stem breast cancer cell line MCF-7 was cultured in the DMEM culture medium containing 10% fetal calf serum at 37℃ and 5% of CO2. When the cell density reached 80%~90%, the passage was carried out, and the logarithmic growth of cells was taken for the subsequent research. The operation was carried out in strict accordance with the instructions for Lipofectamine2000 transfection reagents. The overexpression mimicked miR-138 mimics and the inhibitor anti-miR-138 were transfected into the endometrial stem breast cancer cell line MCF-7, which was set to overexpress miR-138 group and interfere with miR-138, and set up negative control of overexpression and negative control of inhibitor. In the follow-up experiment, the NF-κB agonist was transfected into the miR-138 overexpression group cell, which was set to overexpress miR-138 +NF-κB group.

Test methods

(i) Cell proliferation tested by MTT method: Breast cancer cell MCF-7 at the logarithmic growth stage was taken and inoculated in the 96-well plate for further culture for 12h, and the old culture medium was removed after being attached. Then, the 20μl of 5mg/ml MTT solution was added and cultured for 4h. The supernate was removed, and the 150μl DMSO was added in each group of cells. The supernate was shaken in a constant temperature shock box for 10min. The enzyme-linked immunosorbent assay was used to measure the absorbance values at the 490 m wavelength of each group, and then the proliferation curve was drawn.

(ii) Cell apoptosis experiment: Cells were collected by centrifugation with a non-enzyme digestion solution, and 70% ethyl alcohol was added at room temperature for 5 min. Added PBS and rinsed twice. 100μl 1×binding buffer was used for cell re-suspension; then, PBS, 5μlPI dyeing liquor, AV-FIT Cantibody, PI dyeing liquor +AV-FITC were added and mixed; after that, 400μl 1×binding buffer was added again for cell re-suspension; and the flow cytometer was used to test cell apoptosis.

(iii) Expression of inflammatory factors tested by ELISA method: The cell culture solution was taken out after culture for 48h, and the supernate was collected after centrifugation. The ELISA kit was used

to test the changes of tumor necrosis factor-α (TNF-α), interleukin 1β, 6, 18 (Interleukin1β, 6, 18, IL-1β, IL-6, IL-18) levels.

(iv) Expression of related proteins tested by Western blot method: The logarithmic growth of cells of each group was collected, and the RIPA pyrolysis liquid was added to fully lysate the cells. The BCA method was used to test the protein concentration. The 30μg protein and 4× loading buffer were mixed for centrifugation, then the supernate was taken and transferred to the PVDF membrane after SDS-PAGE gel electrophoresis. 5% of skim milk powder was sealed at room temperature for 1h, and the PVDF membrane was placed into the primary antibody Bax, NF-κB and VEGF for incubation process, and then was incubated overnight in the refrigerator at 4℃. TBST was used to wash the primary antibody that was not specifically bound to the membrane, and the corresponding secondary antibody was added and incubated at room temperature for 1-2h. TBST was used to wash the secondary antibody that was not specifically bound to the membrane. ECL chemiluminescent immunoassay was used for coloration.

Statistical methods

The research data were analyzed by using the SPSS 21.0 software package, and the measured data in accordance with normal distribution were expressed by ($\bar{x} \pm s$). The comparison between multiple groups was conducted by using the one-way variance, and the comparison between two groups with statistical differences would be further carried out with the LSD-t test. When P<0.05, the difference will be deemed to have statistical significance.

Results and discussion

The effect of miR-138 transfection on the proliferation of breast cancer cells

The MTT testing results showed that the proliferation ability of the miR-138 overexpression group was significantly lower than that of the miR-138 overexpression control group (P < 0.05); the proliferation ability of the miR-138 interference group was significantly higher than that of the miR-138 interference control group (P < 0.05). See Table 1

The effect of miR-138 transfection on apoptosis of breast cancer cells

The apoptosis rate, caspase-3 and caspase-9 expression levels of the miR-138 overexpression group were significantly higher than those of the miR-138 overexpression control group (P < 0.05); the apoptosis rate, caspase-3 and caspase-9 expression levels of the miR-138 interference group were significantly lower than those of the miR-138 interference control group (P < 0.05). See Table 2

The effect of miR-138 transfection on inflammatory factors of breast cancer cells

The expression levels of IL-1 β, IL-6, IL-18 and TNF - α in miR-138 overexpression group were significantly lower than those in the miR-138 overexpression control group (P < 0.05); the expression levels of IL-1 β, IL-6, IL-18 and TNF - α in the miR-138 interference group were significantly higher than those in miR-138 interference control group (P < 0.05). See Table 3.

The effect of miR-138 transfection on NF-κB/VEGF signaling pathway of breast cancer

The Western blot testing results showed that the protein expression levels of Bax, NF-κB and VEGF in the miR-138 overexpression group were significantly lower than those in the miR-138 overexpression control group (P < 0.05); the protein expression levels of Bax, NF-κB and VEGF in the miR-138 interference group were significantly higher than those in the miR-138 interference control group (P <0.05). See Table 4

The effect of overexpression miR-138 and NF-κB on the proliferation and apoptosis of breast cancer cells

The MTT test found that the proliferation ability of the miR-138 overexpression group was significantly lower than that of the miR-138 overexpression control group (P < 0.05); the proliferation ability of the miR-138 + NF-κB overexpression group was significantly higher than that of the miR-138 overexpression group (P < 0.05). The apoptosis rate of the miR-138 overexpression group was significantly higher than that of the control group (P<0.05), and the apoptosis rate of the miR-138 + NF-κB overexpression group was significantly lower than that of the miR-138 overexpression group (P<0.05). See Table 5.

Table 1. The Effect of MiR-138 Transfection on Proliferation of Breast Cancer Cells ($\bar{x} \pm s$) (%)

Group	0h	24h	48h	72h
Overexpression control	1.02±0.01	1.03±0.01	1.02±0.01	1.01±0.01
MiR-138 overexpression group	1.01±0.01	0.82±0.02*	0.59±0.02*	0.46±0.03*
Interference control	1.01±0.01	1.02±0.02	1.02±0.01	0.98±0.01
MiR-138 interference	1.01±0.01	1.04±0.04	1.21±0.06#	1.42±0.05#
F	2.50	177.47	655.87	1719.54
P	0.075	＜0.001	＜0.001	＜0.001

Note: Compared with the overexpression control group at the same time point *P<0.05; Compared with the interference control group at the same time point #P<0.05

Table 2. The Effect of miR-138 Transfection on Apoptosis Rate and Factors of Breast Cancer Cells ($\bar{x} \pm s$)

Group	Apoptosis rate (%)	caspase-3	caspase-9
Overexpression control	7.28±1.06	59.67±5.84	20.81±3.26
MiR-138 overexpression	23.97±1.85*	117.56±6.90*	112.31±3.12*
Interference control	10.64±1.25	43.87±5.11	22.54±3.76
MiR-138 interference	4.76±1.54#	19.97±5.32#	15.67±2.35#
F	345.19	506.92	2189.29
P	＜0.001	＜0.001	＜0.001

Note: Compared with the overexpression control group *P<0.05; Compared with the interference control group #P<0.05

Table 3. The Effect of MiR-138 Transfection on Inflammatory Factors of Breast Cancer ($\bar{x} \pm s$)

Group	IL-1β (ng/ml)	IL-6 (ng/ml)	IL-18 (ng/ml)	TNF-α (ng/ml)
Overexpression control	22.52±7.68	31.62±10.39	22.36±5.67	55.42±16.78
MiR-138 overexpression	7.45±2.63*	7.46±2.51*	7.41±1.63*	20.16±7.48*
Interference control	28.36±10.18	25.30±9.63	25.85±8.47	22.48±6.52
MiR-138 interference	55.49±19.63#	79.58±12.30#	55.49±17.63#	78.69±18.20#
F	29.01	105.97	38.84	44.34
P	<0.001	<0.001	<0.001	<0.001

Note: Compared with the overexpression control group *P<0.05; Compared with the interference control group #P<0.05

Table 4. Comparison of Bax of Each Group, NF-κB, VEGF Protein Expression Level ($\bar{x} \pm s$)

Group	Bax	NF-κB	VEGF
Overexpression control	1.02±0.18	0.94±0.12	1.02±0.21
MiR-138 overexpression	0.41±0.23*	0.69±0.10*	0.64±0.20*
Interference control	1.09±0.22	1.04±0.18	1.01±0.24
MiR-138 interference	2.41±0.36#	2.51±0.16#	2.59±0.34#
F	107.78	329.01	117.18
P	<0.001	<0.001	<0.001

Note: Compared with the overexpression control group *P<0.05; Compared with the interference control group #P<0.05

Table 5. The Effect of Overexpression MiR-138 and NF-κB on the Proliferation and Apoptosis of Breast Cancer Cells ($\bar{x} \pm s$)

Group	Proliferation rate (%)				Apoptosis rate (%)
	0h	24h	48h	72h	
Control	1.01±0.01	0.89±0.03	0.96±0.02	0.97±0.03	7.13±1.25
MiR-138 overexpression	1.00±0.01	0.86±0.02	0.81±0.04*	0.71±0.02*	25.14±1.72*
Overexpression miR-138 + NF-κB	1.00±0.02	0.61±0.05*	0.58±0.03*	0.42±0.04*	13.43±1.36*
F	1.67	186.58	378.97	783.10	393.36
P	0.207	<0.001	<0.001	<0.001	<0.001

Note: Compared with the control group *P<0.05

Breast cancer, known as the "pink killer", is the most common female malignant tumor, accounting for 24.2 % of all female cancers worldwide. In recent years, the incidence rate of breast cancer has been increasing year by year. It has biological behaviors, such as distant metastasis and invasive growth, and its pathogenesis has not been completely clear yet, which has a serious impact on women's quality of life (6). miRNA is a type of endogenous non-coding micromolecule RNA fragment, which is specifically paired with the 3'UTR region through the incomplete alkaline complementation way to promote the direct degradation of target mRNA or inhibit its translation. It regulates the expression of post-transcriptional genes, thus participating in cell proliferation, apoptosis, angiogenesis and other biological processes (7). The latest study shows that multiple miRNAs affect the apoptosis, invasion and angiogenesis of breast cancer cells, and may involve in the pathophysiological process of breast cancer. Angiogenesis plays an important role in the pathological process of breast cancer. The growth and metastasis of tumors are closely related to angiogenesis. Under normal circumstances, the angiogenesis factor and angiogenesis inhibiting factor are in a dynamic equilibrium state. But in the pathological state, such as injury and tumor, the angiogenesis factor have advantages, resulting in increased angiogenesis and tumor invasion and metastasis (8). miRNA may be the main controller of angiogenesis, and VEGF can stimulate peripheral angiogenesis, which is highly expressed in breast cancer cells. Cell proliferation and apoptosis play an important role in maintaining the growth and

environmental stability of ectopic endometrial cells, which are regulated by several genes in this process. Among them, miRNA can regulate these genes and plays an important role in the proliferation and apoptosis of breast cancer cells (9). miR-138 is a newly discovered miRNA with tumor regulation in recent years, and it has been verified that miR-138 plays a role in promoting or inhibiting cancer in a variety of tumor tissues (10). Islam M et al. found through their studies that miR-138 can inhibit the proliferation and invasion ability of head-neck squamous cell carcinoma (HNSCC), and can lead to cell cycle arrest and promote cell apoptosis (11). However, there are few reports on the relationship between miR-138 and the biological behavior of breast cancer cells.

In this research, the cell biological functions were studied through the miR-138 overexpression group and breast cancer cell interference models. It was found that the proliferation ability of the miR-138 overexpression group was significantly lower than that of the miR-138 overexpression control group, and the apoptosis rate, caspase-3 and caspase-9 expression levels of the miR-138 overexpression group were significantly higher than those of the miR-138 overexpression control group ($P < 0.05$). In addition, expression levels of IL-1 β, IL-6, IL-18 and TNF - α in the miR-138 overexpression group were significantly lower than those in the miR-138 overexpression control group ($P < 0.05$), which indicated that miR-138 can inhibit the proliferation of breast cancer cells, promote cell apoptosis, and inhibit the expression of inflammatory factors in cells. Some scholars found through the study of cerebral ischemia/reperfusion injury in rats that miR-138 has a protective effect on the inflammatory response caused by injury in rats.

NF-κB is an important nuclear transcription factor, and can be bound to promoters of various cellular genes after activation, which promotes the transcription and expression of target genes, and is closely related to various biological processes, such as cell growth, differentiation and inflammatory response (12). The target gene products of NF-κB, including IL-1β, IL-6 and TNF-α, can activate NF-κB, which plays a positive feedback regulation role, and further enlarges its biological effects (13). VEGF is highly expressed in breast cancer models and tissue samples of patients and is closely related to disease activities and development stages, which be involved in angiopoiesis during the growth and metastasis of breast cancer cells and can be used as a potential therapeutic target for breast cancer (14-16). The research results showed that the protein expression levels of Bax, NF-κB and VEGF in the miR-138 overexpression group were significantly lower than those in the miR-138 overexpression control group ($P < 0.05$). The biological function of miR-138 was restored by using the NF-κB overexpression vector, and it was found that the proliferation ability of miR-138 + NF-κB overexpression group was significantly higher than that of the miR-138 overexpression group ($P < 0.05$), and the apoptosis rate was significantly lower than that of the miR-138 overexpression group ($P<0.05$). This shows that miR-138 may regulate the proliferation and apoptosis of breast cancer cells through NF-κB/VEGF signaling pathway.

To sum up, MiR-138 can significantly inhibit the proliferation of breast cancer cells, promote apoptosis, and regulate the expression of inflammatory factors in the cells. It is speculated that the related mechanism may be related to the negative regulation of the NF-κB/VEGF signaling pathway.

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None.

Conflict interest

The authors declare no conflict of interest.

References

1. Lu X Q, Yang R, Zhang J Q, et al. Effect of farrerol on the proliferation of estrogen receptor-positive breast cancer by downregulating oncogenic genes with estrogen receptor response elements. J Clin Oncol 2020; 38(15_suppl):e12541-e12541.
2. Ming, Wenhui, Zheng, et al. Downregulation of RAI14 inhibits the proliferation and invasion of breast cancer cells. J Cancer, 2019; 10(25):6341-6348.
3. Bai S, Kubelka J, Piri M . Atomistic Molecular Dynamics Simulations of Surfactant-Induced Wettability Alteration in Crevices of Calcite Nanopores. Energy Fuels 2020; 34(3):3135-3143.
4. Jo N, Marcal L, Katabathina V S, et al. Temporal evolution of metastatic disease: part I—an in-depth review of the evolution of metastatic disease across diverse spectrum of non-neural solid tumors on serial oncologic imaging studies and relevant practical applications. Jpn J Radiol 2021;1-19.

5. Sun B, Liu Y, Huang W, et al. Functional identification of a rare vascular endothelial growth factor a (VEGFA) variant associating with the nonsyndromic cleft lip with/without cleft palate. Bioeng 2021; 12(1):1471-1483.
6. Zheng M. Quantitative Analysis for the Spread Range of Malignant Tumor Based on Lie Symmetry. Complexity, 2020; 2020(5):1-6.
7. Li D, Song H, Wu T, et al. MiR-138-5p targeting LIMK1 suppresses breast cancer cell proliferation and motility. Rsc Adv 2017; 7(82):52030-52038.
8. Vs A, Fg A, Dm A, et al. Negative regulation of angiogenesis by novel micro RNAs. Pharmacological Research, 2019, 139:173-181.
9. Huang HT, Wang F, Ding SG, et al. Overexpression of MiRNA-146A promoting the proliferation and angiogenesis of human coronary artery endothelial cells via NF2. Acta Med Mediterr 2019, 35(1):109-116.
10. Erdmann K, Kaulke K, Rieger C, et al. Induction of alpha-methylacyl-CoA racemase by miR-138 via up-regulation of β-catenin in prostate cancer cells. J Cancer Res Clin Oncol 2017; 143(2):1-10.
11. Islam M, Datta J, Lang J C, et al. Down regulation of RhoC by microRNA-138 results in de-activation of FAK, Src, and Erk1/2 signaling pathway in head and neck squamous cell carcinoma. Oral Oncol 2014; 50(5):448-456.
12. Afonina I S, Zhong Z, Karin M, et al. Limiting inflammation-the negative regulation of NF-κB and the NLRP3 inflammasome. Nat Immunol 2017; 18(8):861-869.
13. Grinberg-Bleyer Y, Oh H, Desrichard A, et al. NF-κB c-Rel Is Crucial for the Regulatory T Cell Immune Checkpoint in Cancer. Cell 2017; 170(6):1096-1108.
14. Cosentino G, Stefano V D, Presti R L, et al. Expression pattern of matrix metalloproteinases-2 and -9 and their tissue inhibitors in patients with chronic inflammatory demyelinating polyneuropathy. Neurol Sci 2021:1-4.
15. Tourang, M., Fang, L., Zhong, Y., Suthar, R. Association between Human Endogenous Retrovirus K gene expression and breast cancer. Cell Mol Biomedl Rep 2021; 1(1): 7-13. doi: 10.55705/cmbr.2021.138810.1008
16. Soltész B, Lukács J, Szilágyi E, et al. Expression of CD24 in plasma, exosome and ovarian tissue samples of serous ovarian cancer patients. J Biotechnol 2019; 298:16-20.