

Research on Influences and Molecular Regulatory Mechanisms of miR-193b on Metastasis of Breast Cancer

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Abstract: To investigate the effect of miR -193b on invasion and metastasis of breast cancer cells and its mechanism. MicroRNA array was used to detect the difference of microRNA expression profiles between breast cancer cells MDA-MB-231 and their lung high metastasis potential cell lines (MDA-MB-231-HM). Bioinformatics and other experimental methods are used to find the molecular targets of microRNA. Overexpression of microRNA-193b can reduce the expression efficiency of 3'UTR fluorescent plasmid with uPA. Moreover, microRNA-193b can inhibit the protein expression level of uPA without affecting its mRNA expression level. MiR-193b can inhibit the expression of uPA and aggravate the metastasis and invasion of breast cancer.

1. Introduction

The most common malignant tumors with bone metastasis are breast cancer. The rate of bone metastasis can reach 65%-75%. Paclitaxel (PTX) has significant inhibitory activity against a variety of solid tumors, including breast cancer, advanced ovarian cancer, lung cancer, head and neck cancer and acute leukemia. However, the clinical application of PTX is greatly limited because of its poor water solubility and low bioavailability. After screening, a subclone with high lung metastasis ability was established. They have the same genetic background, but have higher lung metastasis potential. There have also been reports of microRNA and cancer, including breast cancer metastasis. LiMa et al. found that microRNA-10b could also promote breast cancer metastasis, inhibiting microRNA-10b in the highly invasive breast cancer cell line MDA-MB-231, and significantly inhibiting the invasive ability of MDA-MB-231. Overexpression of microRNA-10b in non-invasive breast cancer cell SUM149 significantly increased the invasiveness of SUM149 cells in vivo and in vitro, and the regulation of microRNA-10b on breast cancer metastasis was due to the inhibition of the metastasis suppressor gene HOXD10. The expression of microRNA-10b in metastatic breast cancer tissues was significantly increased. Therefore, comparing the differentially expressed proteins between the two cells can help us to find new prognostic indicators and potential therapeutic targets for breast cancer.

2. Materials and Methods

2.1 Materials.

Cell culture related reagents: DMEM cell culture medium (containing phenol red) purchased from Gibco Company; fetal bovine serum (FBS) purchased from Hangzhou Sijiqing Biological Products Co., Ltd., PBS solvent and trypsin purchased from Gibco Company. Total RNA extraction reagent: Trizol was purchased from Invitrogen Company. Main experimental instruments: carbon dioxide incubator (Shell Lab), microscopy (Nikon), flow cytometry (BD), etc. Animal origin and strain: BALB/c (nu/nu) nude mice, provided by Institute of Medicine, Chinese Academy of Sciences; Qualification Certificate No. 122; Age: 28-30 days; Weight: 16-20g; Gender: Female: raised in SPF environment.

Transwell related reagents: Matrigel and 8insert rooms were purchased from R&D Systems;

Giemsa dye solution was purchased from Beijing Yaoming Foreword Technology Company. CellCounting Kit-8 was purchased from Japan Tongren Institute of Chemistry. Immunohistochemical reagents: Citric acid repair solution (0.01M, pH6.0) was purchased from Shanghai Yusen Biotechnology Company; hematoxylin dye was purchased from Shanghai Hongqiao Lexiang Medical Reagent Company; DAB color reagent kit was purchased from Shanghai Changdao Biological Company; 3% hydrogen peroxide was purchased from Shanghai Biotechnology Company.

2.2 Methods.

The screening of lung high metastasis cell lines will be digested with 0.25% trypsin and suspended in PBS solution to adjust the cell concentration to $1 \times 10^6/\text{mL}$. Under sterile conditions, tumor cells will be inoculated into the fat pad of the left second nipple of nude mice with a 1mL syringe, inoculated with two 0.1mL each, a total of 10. After 8 weeks of culture, nude mice will be killed by anesthesia and immersed in 75% alcohol. 10 minutes: sterile operation, dissection of nude mice, complete removal of soiling, immersed in physiological saline for half a minute; under the microscope, ophthalmic scissors were used to cut lung metastases, and then cut them into 6-well plates for culture; after cell adherence, fresh medium was replaced; semi-digestive and semi-mechanical methods were used to remove fibroblasts: in vitro expansion of selected tumor cells, Some were used for next round screening and some were frozen.

IRNAarray analysis: MDA-MB-231 and MDA-MB-231 cells in logarithmic growth phase (cultured in a 10 cm culture dish) were taken out and washed twice with PBS. PBS was then removed and Trizol 15 minutes of cells were lysed with 2 ml reagent at room temperature. The lysed cells were then moved to the centrifugal tube of Rnasefree by cell scraping and Tip head. Send it to Beijing Capital Bio Company the next day. Capital Bio extracts microRNAs from cells lysed with Trizol, and then compares the microRNAs expression profiles of MDA-MB-231 and MBA-MB-231-1 with that of microRNAarraychip. The extracted microRNAs were hybridized with fluorescent labeled probes containing 435 mature human microRNAs. In order to ensure the reliability of data, each chip is repeated three times. GenePixPro4.0 software was used to homogenize the original data of the chip.

Protein treatment: The cracked protein was mixed with 2 *SDS-PAGE sample buffer in 1:1 volume, and then boiled in boiling water for 5 minutes. Electrophoresis: The coagulated protein gel was put into the electrophoresis device and filled with 1 x protein electrophoresis buffer. The 5u1 protein marker and the same amount of protein samples were injected into the swimming lane of the superstratum gel. Starting with 80 V constant voltage current electrophoresis, when the sample enters the separation gel, the voltage is changed to 100 V. When bromophenol blue completely escaped from the gel, the electrophoresis was stopped. Transfer Membrane: Cut off the PVDF membrane, so that the size of the PVDF membrane is the same as the size of the filter paper. Then the PVDF membrane is immersed in methanol for 20 seconds, and then immersed in the balance of the electro-transfer buffer. The gel was stripped from the glass plate and placed in the film transfer solution. According to the order of negative electrodes, two filter paper, gel, PVDF membrane, two filter paper and positive electrode, after the alignment of the electrode, insert into the film transfer frame, in the ice trough, with the constant flow film of 200mA.

The logarithmic growth of breast cancer cells was adjusted to $2 \times 10^6/\text{ml}$ sterile condition. Tumor cells were inoculated into the fat pad of the left second papilla of nude mice with a dose of 0.1 M1 by microinjector. Six nude mice were inoculated into each group. The health status of nude mice and the tumorigenicity of tumor cells were observed every other day. After tumorigenesis, the volume of transplanted tumors was measured every three days (volume: $1/2ab^2$, A and B were the length and short diameter of transplanted tumors). At the eighth week, nude mice were executed, lung tissues were removed and inoculated with formalin solution for fixation; lung tissues were sent to the case Department of Fudan University for serial sections and HE staining to observe the formation of pulmonary metastases; and inoculated tumors were also sent to the case Department of Fudan University for paraffin embedding and white sections.

2.3 Results

MDA-MB-231-HM cells were established after six rounds of screening of wild-type MDA-MB-231 cells, which had high potential for lung metastasis. The tumorigenesis rate of wild type MDA-MB-231 and MDA-MB-231-HM cells was 100% within four weeks after inoculation, but the rate of metastasis in lung of wild type MDA-MB-231 cells was less than 50%, but that of MDA-MB-2314M cells was 100%. By comparing the microRNAs expression profiles of MDA-MB-231 and MDA-MB-231-HM, 80 differentially expressed microRNA0 were screened, with the false positive rate set below 5%, and 19 differentially expressed microRNAs were obtained.

PGL3-control-uPA-3'UTR plasmid, pRL-C plasmid, pRL-C plasmid and RNA-193b expression plasmid were transfected into MDA-MB-231 or MDA-MB-435 cells at the same time. Overexpression of microRNA-193b could significantly reduce the expression efficiency of fluorescent plasmid with uPA-3 and UTR; pGL3-control-uPA-3'UTR plasmid, pGL3-control-uPA-uPA-3'UTR-3'UTR plasmid, pRL-CMV plasmid and the inhibitor of microRNA-193b were transfected into T47D47Dcells at the same time. When the microRNA-193b was suppressed, the microRNA-193b was inhibited. The results of this study are as follows:1. The expression efficiency of the plasmid increased significantly (0.05). After mutation of the predicted site of microRNA-193b on uPA-3'UTR, the mutant pGL3-control-uPA-3'UTR plasmid, pRL-CMV plasmid and the expression plasmid of microRNA-193b were transfected into MDA-MB-231 or MDA-MB-435 cells simultaneously. It was found that the inhibitory effect of microRNA-193b on fluorescent plasmids with uPA-3 and UTR disappeared after mutation of the site. However, this change was not obvious in MDA-MB-231 cells.

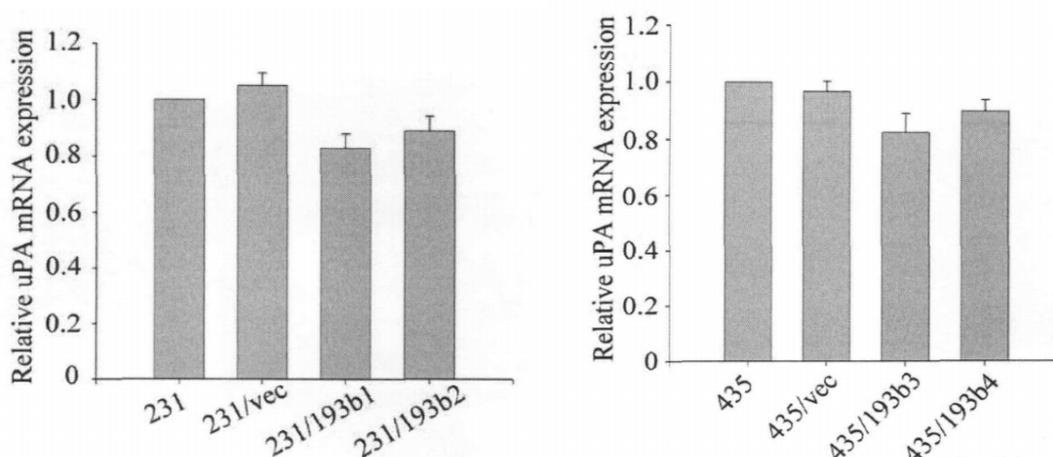


Figure 1. Relative uPA mRNA expression in MDA-MB-231cell (left) and MDA-MB-231 cell (right)

2.4 Discussion

Many experiments have been carried out at home and abroad to screen subclonal breast cancer cell lines with higher metastasis ability in nude mice by human breast cancer cells. Proto-cell lines and screened subcell lines have the same genetic background. By comparing the differences of molecular expression profiles between proto-cell lines and sub-cell lines, it is possible to find molecules that have important relationship with breast cancer metastasis. This provides very important information for studying the molecular mechanism of breast cancer metastasis and the treatment of breast cancer. This project is also the first to screen subclones of breast cancer cell line MDA-MB-231 with stronger metastatic potential. By comparing the differences of RNA expression profiles between the two cell lines through microRNAarray, it is also a unique feature of this topic to find clinical microRNA0 related to breast cancer metastasis.

Because microRNAs can only play a biological role by regulating protein-coding genes, we analyzed the bioinformatics of 19 differentially expressed microRNAs. Our entry point is that if a candidate target of microRNA has been confirmed to be closely related to the metastasis of breast

cancer, then if we verify the regulation of this microRNA on this candidate target, then this microRNA is also microRNA0uPA and has-microRNA-193b related to breast cancer metastasis, which arouses our great interest. Firstly, compared with MDA-MB-231 cells, the expression level of microRNA-193b in MDA-MB-231-HM was significantly down-regulated. Secondly, three software systems, TargetScan, PicTar and MicroRNAda, were used to predict that hsa-microRNA-193b had a common role in human uPA-3 and UTR. Finally, uPA was closely related to the metastasis and recurrence of breast cancer, and the high expression of LIPA could promote the metastasis of breast cancer. Move. The above prediction has been verified. Compared with MDA-MB-231 cells, the expression level of microRNA-193b in MDA-MB-231-HM was significantly down-regulated; the expression level of uPA was basically unchanged; the protein expression level of uPA was significantly increased.

In order to verify the regulatory effect of microRNA-193b on uPA, the 3'UTR of uPA is positively located between the luciferase coding region and PO1YA sequence of pGL3-controlvector. The recombinant pGL3-controlvector transcripts the 3', UTRO3, UTR-uPA-luciferase, internal reference plasmid pRL-CW and microRNA-193b inhibitors into T47D cells. When the endogenous microRNA-193b of T47D cells is suppressed, the fluorescent plasmid is transfected. The expression efficiency increased significantly. At the same time, over-expression of microRNA-193b in MDA-MB-231 and MDA-MB-435 cells, and over-expression of microRNA-193b significantly reduced the expression efficiency of fluorescent plasmids. When the predicted binding site of microRNA-193b on uPA 3' and UTR was mutated, the expression efficiency of fluorescent plasmids was restored. This indicates that mir-193b can directly regulate LIPA. Moreover, microRNA-193b can inhibit the transcriptional level of endogenous uPA. It is speculated that the reasons are as follows: a. MDA-MB-231 is a cell with high expression of uPA, and MDA-MB-231 is a low expression of uPA. Therefore, in vivo experiment, microRNA-193b could not inhibit o1st-Hanseneal. and MDA-MB-231 cells. B. Microamounts of uPA in stromal cells of nude mice also promote the proliferation and invasion of inoculated tumor cells in nude mice. In vivo experiments of MDA-MB-435 also showed that uPA was an important downstream target for the biological function of microRNA-193b to some extent. In vivo experiments on MDA-MB-435 cells also show that uPA is an important downstream target for the biological function of microRNA-193b. The biological function of microRNA-193b is mainly through inhibiting uPA.

Chemotherapy or radiotherapy were not explained in 80 cases of breast cancer before operation. The results showed that there were significant differences in the expression of microRNA-193b between positive and negative lymph node metastasis patients: the overall expression level of microRNA-193b in 40 lymph node-positive patients was significantly lower than that in 40 lymph node-negative patients; the relative expression level of microRNA-193b was divided into four groups, and patients with low expression level of microRNA-193b were more likely to have lymph node metastasis. Migration: Mi-193b was not significantly correlated with ER and PR status, but was significantly correlated with tumor grade and stage. Eighty breast cancer specimens selected from the pathological Department of the hospital were divided into two groups according to the positive and negative lymph node metastasis. The expression level of mir-193b in lymph node-positive patients was much lower than that in lymph node-negative patients detected by real-time PCR, and the expression level of uPA antigen in lymph node-positive patients was much higher than that in lymph node-negative patients detected by immunohistochemistry. These data suggest that microRNA-193b is a negative regulator of uPA in breast cancer. In the process of breast cancer metastasis, the decrease of the expression of microRNA-193b is an important reason for the high expression of LIPA. The expression of microRNA-193b is also closely related to the prognosis of breast cancer patients. Patients with high expression of microRNA-193b have better prognosis, while patients with low expression of microRNA-193b have worse prognosis. Mir-193b may be a molecular marker for the prognosis of breast cancer.

3. Conclusion

In this study, we used breast cancer cell line MDA-MB-231 to screen subcellular lines with higher metastasis potential in nude mice as a starting point, and screened the gene mir-193b closely related to breast cancer metastasis. Mir-193b not only inhibits the metastasis of breast cancer, but also inhibits the expression of uPA. Inhibition of uPA expression by microRNA-193b is the main mechanism of its inhibition of breast cancer metastasis. The findings of this study can be summarized into two points: ImiR-193b can inhibit the expression of uPA, a metastasis-promoting gene in breast cancer. During the development of breast cancer, the decrease of the expression of microRNA-193b is an important reason for the high expression of uPA, and also a factor contributing to the metastasis of breast cancer.

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