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Study on the Effect of PIWIL2 Expression on EMT of Breast Cancer Stem Cells and Its Mechanism

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Abstract: Objective: This study mainly elaborated the specific molecular mechanisms of breast cancer metastasis and EMT, which has important clinical value in judging the prognosis and treatment of breast cancer patients. Methods: A total of 493 patients with breast cancer were studied. The expression of PIWIL2 gene was analyzed by immunohistochemistry and section analysis, and the correlation between gene expression and clinicopathological parameters and prognosis of the patients was investigated. TGF-β1 was used to induce breast cancer cells, and EMT model was constructed at the same time to compare the changes of EMT model cells in the control group and obervation group. The expression of PIWIL2 gene in EMT model was detected by Western bloting. After transfection and RNA interference, PIWIL2 expression was silenced. At the same time, the morphological changes of the cells after the reduction of PIWIL2 gene expression were observed through microscope. The gene changes related to EMT were detected by RT-PCR and Western bloting. The expression of PIWIL2 gene was decreased by invasion test. And the expression of breast cell invasiveness and other related expressions such as MMP-13, MMP-9, VEGF shall also be decreased. The effect of MAPKERK and P13K/AKT pathway on the regulation of PIWIL2 expression was studied by obseving the cellular mophology through the microscope. And RT-PCR and Western bloting were used to detect the expression, including Snail, Vimentin and SIug. The effects of PIWIL2 and the downstream transcription factor SIug in ER pathway in breast cancer cells were analyzed by light microscopy, RT-PCR, Western bloting and Transwell invasion experiment. Results: PIWIL2 expression was negatively correlated with its survival rate in estrogen receptor a negative patients, while was not directly related to estrogen receptor positive patients. After the induction of TGF-β1 in MCF-7 breast cancer cells, the cells were spindle shaped and would lose the intercellular adhesion. With the development of EMT, the inhibition of PIWIL2 gene expression would block the effect of TGF-β1 on EMT in MCF-7 cell lines. The changes of EMT-related genes expression can be shown by RT-PCR and Western Bloting. SiRNA sclienced the Piwi12 expression, which will decrease the cellular invasiveness. At the same time, such matrix metalloproteinase as MMP9 and MMP13 and the mRNA transcription vascular endothelial growth factor was significantly down-regulated. After silencing the expression of SIug, all EMT of TGF-β1 gene will be inhibited, and the estrogen a signal pathway can inhibit the expression of PIWIL2 and the downstream transcription factor SIug. Slug is a common downstream transcription factor of estrogen a and PIWIL2, which also serves as an important bridge connecting estrogen a and Piwi12. It can accept Piwi12 and estrogen a to inhibit or stimulate signals, and then effectively regulate the EMT of breast cancer cells. In addition, ER signal can also participate in the expression of Piwi12 and antagonize Piwi12 to promote EMT.

Keywords: PIWIL2; Breast Cancer Stem Cells; EMT Mechanism

1. Introduction

Invasive breast cancer, as a malignant tumor, will greatly affect the health of patients with a incidence of 8%. In recent years, the number of breast cancer patients in China has gradually increased. After standardized surgical treatment and radiotherapy, the mortality rate of breast cancer patients still reached 15%. For breast cancer patients, cell metastasis is the direct cause of death, so an in-depth exploration on the metastasis mechanism of breast cancer cells is of great significance for clinical cure and treatment[1]. EMT refers to the epithelial cells which will lose the polarity after patial stimulation and the adsorption and tight connection between cells, thus obtaining the ability of migration and infiltration, which is specifically manifested as interstitial characteristics, and is also the morphology of embryonic development[2]. In clinical practice, epithelial cells can obtain mesenchymal cell activity through EMT, and after losing the polarity of epithelial cells, maternal cell-like symptoms will appear, and then first migrate to the epithelial cortex and enter the mesoderm to form different tismoids, and further participate in embryonic development. At the same time, it can also participate in tissue regeneration, fibrosis, tumor metastasis, and play an important role in distant metastasis of tumor cells as well as in-situ metastasis and invasion.

2. Expression significance of PIWIL2 in clinical specimens

2.1 Research materials and methods

In this study, 493 patients with breast cancer, were selected as research objects, with an average age of 45 years. The patients chosen were those who did not receive radiotherapy, chemotherapy, or endocrine therapy before surgery. The reagents and drugs used include 3% hydrogen peroxide, PIWIL2 antibody, and DBA chromogenic agent. The instruments used include thermostat, slicer, microscope, refrigerator and so on. In this study, sections were prepared by immunochemical staining of genes, and were stained to evaluate the PIWIL2 protein. The staining range and intensity of the protein could be divided into 3 levels: when the index of PIWIL2 staining coefficient was greater than 8, it represents high expression; and when the staining coefficient of PIWIL2 staining protein was 0, the expression was negative; and when the staining coefficient of PIWIL2 protein was 1-7, it represents low expression.

2.2 Research results

Among the above 493 breast cancer tissue sections, 278 cases of invasive breast cancer tissues showed weakly positive and negative in negative in PIWIL2 protein, which referred to low expression; while 215 cases of breast cancer tissue sections showed strongly positive, which reffered to high expression. The positive expression was found in the nucleus of tumor cells (see Figure 1).

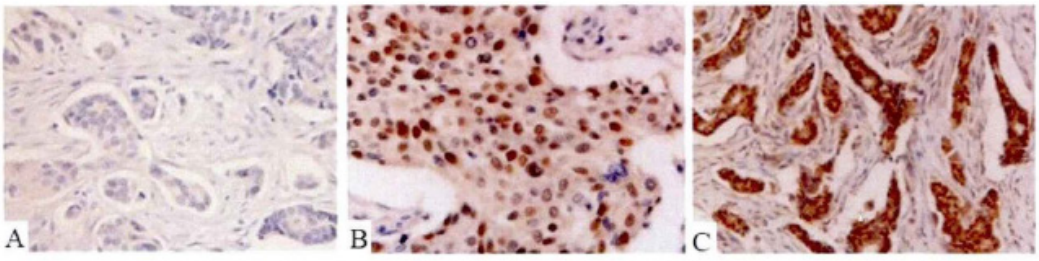

Figure 1. Expression of Piwil2 protein in invasive breast carcinoma

As is shown in the above figure, PIWIL2 protein is weakly positive and negative in breast invasive carcinoma tissues, and its expression level is low. Thus we can infer that the PIWIL2 protein expression is related to the parameters of breast invasion. According to different demerit points, the patients can be grouped into postmenopausal group and premenopausal group in combination with the pathological parameters of breast cancer or according to the menstrual state of the patients[3]. According to tumor size, they were divided into two groups: one group is tumor size less than 2

cm and the other is tumor size more than 2 cm; according to lymph node metastasis, they were divided into negative group and positive group; according to tumor histology, they were divided into group 1, group 2 and group 3. The results showed that the high expression of PIWIL2 protein is associated with the expression relationship, tumor analysis, distant metastasis, and cytological grade of HER2 and ER, while having no significant relationship with the tumor size, age, menopausal status, lymph node status, and clinicopathological indicators of the patients. In the tissue samples of 463 breast cancer patients without distant metastasis, their PIWIL2 expression was negatively correlated with their survival rate.

3. Establish EMT model and analyze the regulatory effect of PIWIL2 in EMT

3.1 Research materials and methods

In this study, breast cancer MCF-7 cell lines were selected for existing culture, which are cultured in a carbon dioxide incubator at 37℃. At this time, cells could be adherent culture and growth reagents including fetal bovine serum, DMSO and 0.25% trypsin, distilled water, Slugj antibody, Snail antibody, PIWIL2 antibody, PVDF membrane, chemical luminescent agent, etc. can be used to promote the adherent culture. The instruments used include cell counting board, super table, electronic balance, ordinary refrigerator, microscope, etc. In the specific operation, cell culture was required, including resuscitation of cells, cell passage, induction experiment, cell protein extraction after cell collection, RNA extraction by Matrigel experiment and RT-PCR.

3.2 Research results

The following figure (Figure 2) shows the cellular morphology of MCF-7 cells induced by TGF-β1. According to the results, it can be found that, compared with the control group, the cell morphology of MCF-7 cells can be significantly changed after 48 hours with 5ng/mlTGF-β1. The whole cell presents long spindle type, and the cells are relatively scattered, and the adhesion between cells is lost, which are characteristic changes of EMT. After adding 5ng/MTGF-β1 treatment, the most obvious changes of cell morphology were observed 96 h later. To verify the presence of EMT, the expression of related proteins can be detected by Western Bloting, as is shown below.

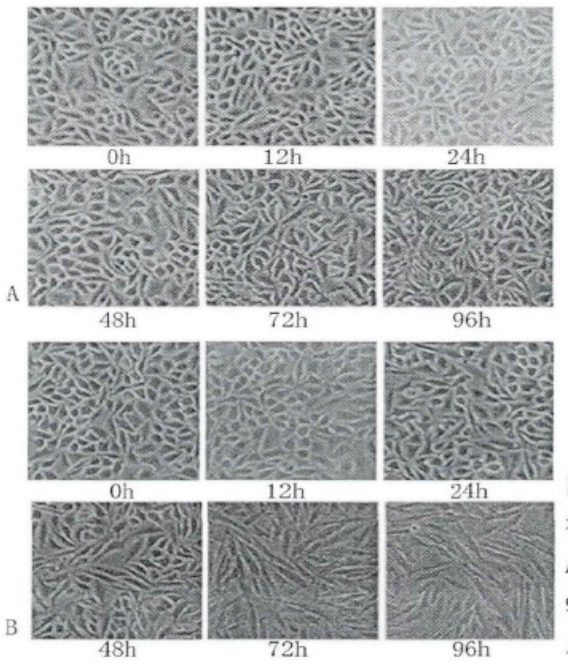

Figure 2. Cell morphology of MCF-7 cells induced by TGF-β1

As can be seen from the above figure, 72h after treatment with LTGF-β1, the expression of E-cadherin and E-acdherin epithelial cell marker protein decreased, both of which are closely associated with EMT and can be considered as characteristic changes of EMT. In addition, the expression levels of nuclear transcription factors Slug, Snail and Fibronecitn significantly increased, which was consistent with the characteristics of EMT, while the expression levels of PAAWI L2 were not significantly changed. The effect of silencing PIWIL2 on key indicators of EMT can be achieved by silencing PIWIL2 expression through SiRNA. After TGF-β1-induced EMT-related gene detection, the cell morphology was observed by microscope. The results are as follows. When dealing with 5ng/mlTGF-β1, the expression of E-cadherin and the epithelial marker protein E-acdherin will reduce after 72 h, both of which are closely associated with EMT and can be considered to be characteristic changes of EMT. In addition,

the expression levels of nuclear transcription factors Slug, Snail and Fibronecitn were significantly increased, which was consistent with EMT characteristics, while the expression levels of PIWIL2 were not significantly changed. The effect of silencing PIWIL2 on key indicators of EMT can be achieved by silencing PIWIL2 expression through siRNA. After EMT-related gene detection induced by TGF-β1, the cellular morphology was observed through microscope. The results are as follows. After induction by TGFβ1 at 5 ng/ml for 48 h, MCF7 in breast cancer will exhibite the morphologic changes of the characteristics of EMT, with cells in long shuttle type and will lose the cellular adhesion. Western bloting was used to detect EMT-related gene expression. The results showed that Vimentin and Snail expression in MCF-7 cells were increased 48 h after stimulation with TGF-β1 per milliliter. At the same time, the expression level of E-cadherin was decreased, and there was no significant change in the expression level of PIWIL2, which was consistent with previous studies. Inhibition of PIWIL2 expression by siRNA can reduce the expression of PIWIL2, and decrease the expression levels of plasmid protein Vimentin, Fibroectin, nuclear transcription factors Slug and Snail. Compared with TGF-β1 group, the expression level of E-cadherin increased. But it was significantly lower than the control group. The results of RT-PCR showed that after induced by TGF-β1 for 48 hours, Piwi12 transcription was not significantly changed, while E-cadherin transcription decreased, and E-cadherin transcription was significantly increased after silencing of Piwi12 gene, indicating that Piwi12 gene expression was silenced, which will inhibit the effect of EMT induced by TGF-β.

4. Regulation mechanism of ERE signal and Piwi12 on EMT

4.1 Research materials and methods

The reagents used in this study include DMSO, transforming growth factor, PIWIL2 antibody, and 0.25% trypsin. The equipment used include electronic balance, cell culture flask, adjustable pipette, electrophoresis equipment, ordinary refrigerator, electrotransfer membrane meter, digital display PH meter, high speed refrigerated centrifuge, etc. In the specific operation, cell proteins were extracted, and liposome-mediated eukaryotic cells were detected by Western Blotting. After transfection of PIWIL2 gene, RNA was extracted and analyzed by RT-PCR.

4.2 Research results

PIWIL2 canal was transiently transfected into MCF-7 cell lines to overexpress PIWIL2 expression in cells by RT-PCR, as is shown in the following figure (Figure 3).

According to the results, the transcription level of mRNA in PIWIL2 was significantly increased after the transfection of PIWI12 cDNA in MCF-7 cells. At the same time, the transcription of Mrna in Slug also increased. Silencing the expression of Piwi12 gene with siRNA can reduce the expression of Slug. Combined with previous studies, it was found that Piwi12 expression in breast cancer samples without distant metastasis was negatively correlated with disease-free survival rate and overall survival rate, and this correlation was directly related to ER expression. In ER negative patients, the expression of Piwi12 gene was inversely proportional to the survival rate of patients, while not directly relating to ER positive patients. Therefore, it can be inferred that ER signal is correlated with Piwi12 expression and biological activity. Western Bloting results showed that the transcription of MCF-7 in breast cancer could further increase the expression of Piwi12 gene while reducing the expression of E-cadherin and increasing the expression of vimentin and Fibronectin. It is proved that Piwi12 can promote the occurrence of EMT.

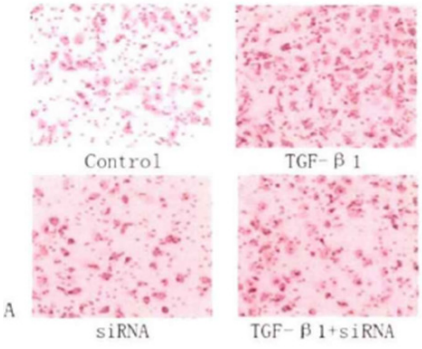

Figure 3.The overexpression of PIWIL2 by RT-PCR when transiently transfecting into MCF-7

After E2 activates the ER pathway, the expression of Slug and PIWIL2 will be restricted, while the expression of E-cadherin is up-regulated, the

expression of vimentin and Fibronectin will decrease. In addition, the transcription of Slug, E-cadherin and PIWIL2 is compared by RT-PCR, and the results show that the transfection level of PIWIL2 gene and the Slug transfection will reduce correspondently. E-cadherin, as an indicator of EMT epithelial gene, when increasing its transcription level, it will indicate that E2 or ER signals could effectively antagonize PIWIL2 and promote EMT generation. As PIWIL2 regulates EMT, ER can participate in it. According to the combined study, the downstream gene of PIWIL2, Slug, is also a co-transcriptome of EMT. It is speculated that the PIWIL2 gene plays an important role in the participation of ER signal, and in fact, the Slug gene can be silenced by Slug transfer into the molecule, as shown in the figure below. The expression of Slug gene was inhibited, which would decrease the expression of PIWIL2. In addition, EMT is an important part-time indicator, which can decrease the gene expression of Fibronectin and Vimentin, and meanwhile increase the transcriptional expression level of E-cadherin in epithelial gene indexes. Combined with RT-PCR results, it was found that the transcription level of E-cadherin in TGF-β1 group was decreased, while the transcription level of Slug was increased. After the silencing of Slug gene, the transcription of E-cadherin wil significantly increase compared with that in TGF-β1 group.

4.3 Disscussion and conclusion

In combination with studies, it was found that long-term exposure to E2 would increase the incidence of breast cancer in patients with different cancer-promoting effects, and ERA was considered to have a negative correlation with metastasis and invasion of breast cancer cells[4]. At the same time, ERA is also an independent prognostic factor of breast cancer. Negative expression of ERA will lead to poor prognosis of patients, with different effects for different patients. When patients with positive breast cancer receive post-operative anti-estrogen therapy, their cancer sells may inhibit metastasis during breast cancer treatment by antagonizing[5]. Analysis of ERA signal pathway to understand the relationship between breast cancer cell metastasis and ERA signal can provide a reference for clinical treatment. Finally, the study showed that in breast cancer cells, the expression of PIWIL2 gene was inversely proportional to the overall survival of patients. Therefore, PIWIL2 can be used as an important marker of biological behavior of breast cancer invasion, which is closely related to the prognosis of breast cancer patients. It also indicates that the expression of PIWIL2 itself and downstream transfer factors as well as biological activity are regulated by ER signal pathway.

In conclusion, this paper analyzed the specific role and regulatory mechanism of PIWIL2 in breast cancer. As a nuclear transcription factor, PIWIL2 can accept TGFβ-induced signals and control the invasion and metastasis of breast cancer cells by mediating the expression of various downstream transcription factors and related proteins. EMT is a prerequisite for breast cancer metastasis, which is also an important link of cancer cell metastasis and plays a role in the expression of PIWIL2 gene in breast cancer. TGFβ-induced EMT process can be limited by down-regulating PIWIL2 expression. However, due to the relatively complex processing mechanism of EMT, further investigation is needed to improve the conclusion.

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