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Original Article

TGF-β1 gene silencing can enhances the sensitivity of breast cancer to cisplatin partially by restraining the occurrence of EMT

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Abstract: Breast cancer is one of the most common gynecological malignant tumors, the main reason of treatment failure is distant metastasis and local recurrence. TGF-β1 as a versatile polypeptide molecule plays an important role in inducing EMT to promote tumor invasion and metastasis. This study aims to investigate the effect of TGF-β1 on cisplatin (DDP) inhibiting the proliferation, migration and invasion of breast cancer and its correlation with EMT. TGF-β1 siRNA were transfected into MCF-7 cells. The cell morphology, proliferation, migration and invasion ability changes were detected by inverted microscope, clone formation assay, cell adhesion assay and Transwell Chamber Invasion. The expression of E-cadherin, vimentin, α-SMA were detected by Western blot. The results showed that TGF-β1 siRNA were transfected into MCF-7 cells successfully ($P<0.05$). The inhibitory activity of cisplatin on cell proliferation, migration and invasion of breast cancer were significantly enhanced after TGF-β1 siRNA transfection ($P<0.05$). The expression of E-cadherin was up-regulated, and vimentin and α-SMA were down-regulated with TGF-β1 siRNA transfection ($P<0.05$). Therefore, we concluded that TGF-β1 gene silencing can enhance the sensitivity of breast cancer to cisplatin on proliferation, migration and invasion partially by restraining the occurrence of EMT.

Keywords: Breast cancer, TGF-β1, EMT, siRNA, proliferation, migration, invasion

Introduction

Breast cancer is one of most frequent malignant tumors among the female, and its incidence rises up year by year. And the distant metastasis and local recurrence of tumor are the main reasons leading to the failure of treatment for breast cancer, exploring a new and effective therapeutic modality is to solve this urgent problem. Epithelial-mesenchymal transition (EMT) is a complex dynamic process including loss of intercellular junction, change of polarized epithelial cell, skeleton reconstruction, obtaining of mesenchyme, and other biological behavior. Such process is closely related to tumor invasion and transfer process as it can change the cell adhesion and cellular morphology, and stimulate relevant signal pathways related to cancer occurrence. Recent study showed that EMT is one of the key factors accelerating the occurrence and development as well as the recurrence and metastasis of breast cancer [1].

TGF-β, as a versatile polypeptide molecule, can be divided into four subtypes including TGF-β1, TGF-β2, TGF-β3 and TGF-β1β2. More specifically, TGF-β1, accounting for the largest proportion, is mainly expressed in endothelial cells, hematopoietic cells, and cells connecting to the tissues. It plays a key role in cell proliferation and differentiation, embryonic development, adhesion migration, vascularization, would healing [2]. Over the recent years, relevant studies show that TGF-β1 is highly expressed in many solid tumor, such as gastric cancer, intestinal cancer, cervical cancer, and breast cancer and closely related to the occurrence and drug resistance of tumor, tumor-related angiogenesis, as well as tumor cell invasion and metastasis. It stimulates the occurrence and development of breast cancer and is one of the key

factors of bad prognosis of breast cancer. TGF-β1 mainly promotes the infiltration and metastasis of breast cancer by inducing angiogenesis and triggering epithelial-mesenchymal transformation, and plays its role by affecting the downstream protein molecules. Research also found that the silent TGF-β1/Smad 4 signal path can strengthen the lethal effect of chemotherapy drug on tumor. It has chemosensitive effect, which is a breakthrough of the research [3].

Thus, in our study, we select breast cancer MCF-7 cells as cell models, and investigate the effect of cisplatin on cell proliferation, migration, and invasion after TGF-β1 siRNA transfection.

Materials and methods

Cells and reagents

Human breast cancer MCF-7 cell lines were purchased from the Institute of Cryobiology, Chinese Academy of Sciences. RPMU1640, fetal bovine serum and trypsase all were bought from Gibco (USA). Cisplatin (DDP), batch number: D20170183, Qilu Pharmaceutical Co., Ltd. 8 m aperture matrigel was purchased from RiboBio Bio-chemistry Ltd. The primers sequence of TGF-β1 siRNA was designed by PCR primer design software Primer Premier 5, and synthesized by Invitrogen Company. TGF-β1 siRNA: 5'-GCAGAGTACAC ACAGCATA-3'. Rabbit anti-human TGF-β1, E-cadherin, Vimentin, α-SMA and mouse anti-human β-actin polyclonal antibody were purchased from Abcam, U.S. The light microscope was purchased from Nikon Co., Ltd. Japan.

Cell proliferation was detected by MTT

The cells in the logarithmic growth phase in each group were digested and counted. Different concentrations of DDP (0, 0.5, 1, 2, 4, 8 μmol/L) were adopted for cell processing. There were 3 multiple pores in each group. After 48 h of culture, 10 μl of MTT solution (concentration: 5 g/L) was injected into each pore for 4 h of continual incubation, discard the supernatant; 150 μl of DMSO was added into each pore under 37°C of incubation for 30 min. Full-automatic quantitative mapping microplate reader was adopted to measure the absorbance A of 570 nm wave length of each pore. Repeated experiment of each drug was adopted for 3 groups. Linear regression was adopted to calculate half maximal inhibitory concentration (IC50).

siRNA tranfection efficiency was detected by Western blot

Inoculate cells of logarithmic phase into 24 pores, each pore 1×10^5 cells. After 24 h of culture, discard the culture solution, wash with PBS for one time. Mix different volume of lipidosome (Lipofectamine 2000, Life Technologies) with 50 μl opti-MEM; mix 1 μl of negative control FAM, NC-FAM (5'-UUCUCCG AACGUGUC-ACGUTT-3') and 50 μl of opti-MEM, stand for 5 min; add Lipofectamine 2000 into NC-FAM, and make compound according to the proportion of Lipofectamine 2000: NC-FAM=1:4, 1:2, 1:1, 2:1, and 5:2, stand for 20 min; add the compound into each pore, and add opti-MEM so that the final pore of each pore is 2 ml. After 12 h, observe the transfection efficiency under fluorescence microscope and select 1:1 as the optimal proportion. Make the cells of logarithmic phase into single-cell suspension and inoculate them to 6-pore plate at 4×10^5 cells/each pore. After 24 h of culture, transfect them at 1:1 ration. After 6 h, replace the original solution with fresh medium. Collect the cells for protein extraction on the next day. Detect the influence of TGF-β1 siRNA on TGF-β1 protein expression in the MCF-7 cells of breast cancer.

Cells culture and grouping

The human breast cancer MCF-7 cells were cultured in the RPMI-1640 culture medium (containing 10% of the fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) in 37°C, 5% CO_2 and saturated humidity incubator. The cells were used in the experiment when they were in the logarithmic phase. The cells were divided into control group, DDP processing group, and DDP+TGF-β1 siRNA group.

Observation of cellular morphology under inverted microscope

After 48 hours of culture, the morphologic change of cells of three groups was observed and pictures were taken picture with inverted microscope.

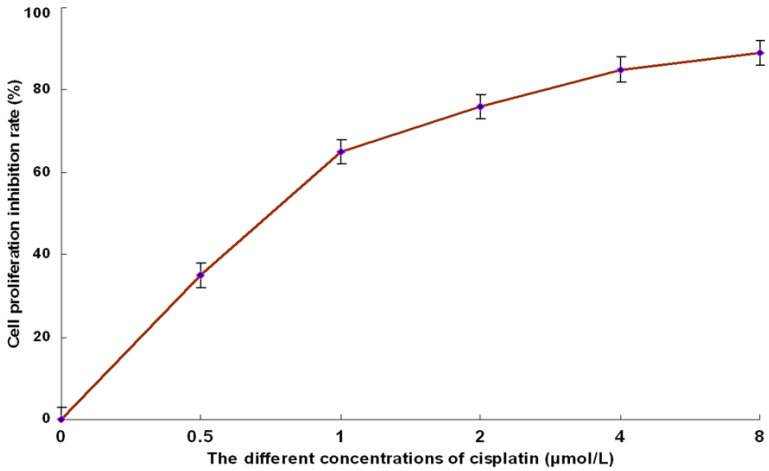

Figure 1. Effects of DDP on proliferation of MCF-7 cell lines.

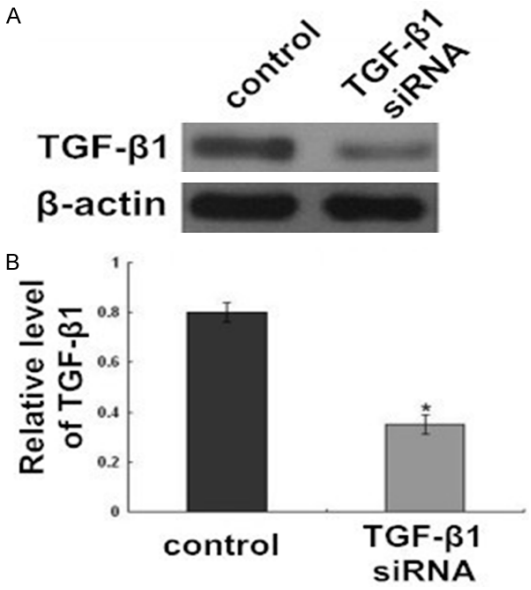

Figure 2. Effect of TGF-β1 siRNA on the expression of TGF-β1 protein in MCF-7 cells detected by Western blot. A: Protein blotting stripe. B: The relative level of TGF-β1 protein. *P<0.05 versus the control group.

Cloning activity was detected by single cell clone formation assay

When digesting MCF-7 cells with pancreatin, inoculate them into 6-pore plate at 200 cells/pore, disperse the cells. When cell clone (over 50 cells are regarded as a clone) occurs in the pore plate, terminate the culture. Discard the supernatant, and wash with PBS for 2 times. Fix it with 4% paraformaldehyde for 10 min, and wash it with PBS for three times. Crystal violet staining for 20 min, and wash it for 3 times. Observe the control group and experiment group with inverted microscope, four fields of view in total, calculate the numbers of cell clone and take photos.

Cell migration was detected by cell wound scratch assay

The cells in the logarithmic growth phase in each group were digested and counted. And inoculate them into 6-pore plate at 5×10^5 cells/pore and mix well. When cells are grown to 90% for mixing, 200 μl of sterile spearhead was adopted for vertical scratching at a line space of 1 cm. PBS was used to rinse the floating cells. Place the floating cells in 5% of CO_2 under 37°C and saturated humidity for continual culture. Observe the scratch healing status of cells and take photos at 0, 24 h, 48 h, and 36 h respectively.

Cell invasion was detected by transwell chamber invasion

The cells in the logarithmic growth phase in each group were digested and counted. On the upper Transwell chamber that was evenly paved with Matrigel, 200 μL of non-serum culture medium that contained 5×10^4 cells was added. The Mixture was then incubated in a 5% CO_2 incubator at 37°C for 24 hours. As the incubation finished, the cells were taken out and washed twice with PBS then fixed with 4% paraformaldehyde for 10 minutes. Afterwards, another 2 PBS washes were given before the cells were dyed with crystal violet for 15 minutes. Following another 2 PBS washes, the cells on the upper chamber were carefully wiped away with cotton bud. Eventually, pictures were taken in 8 random views under microscope for records.

Expression of E-cadherin, vimentin and α-SMA was detected by Western blot

The cells in the logarithmic growth phase in each group were digested and counted. Afterwards, the culture medium was discarded whereas the cells were washed 3 times with

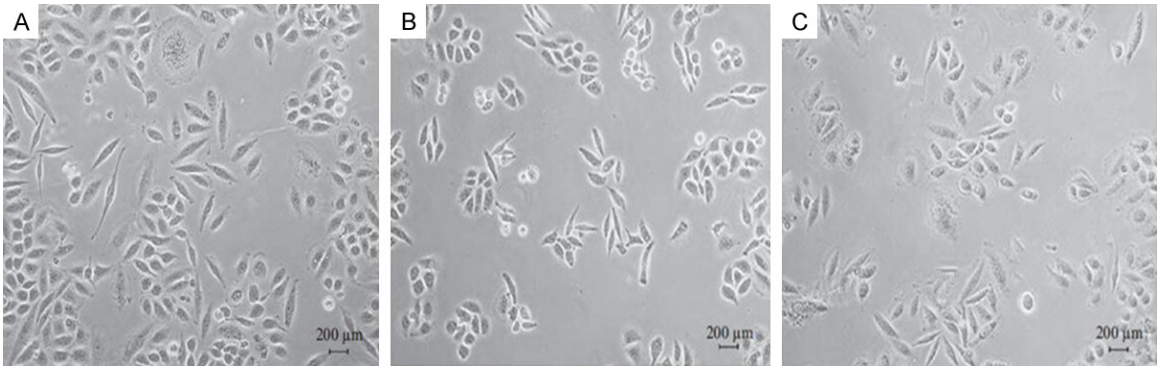

Figure 3. The morphology of MCF-7 cells under inverted microscope (1:200). A: U2OS/DDP cell lines; B: U2OS/DDP/TUSC7 cell lines; C: U2OS/DDP/TUSC7 cell lines after TGF-β1 siRNA transfection.

PBS. 150 μl of pre-chilled RIP A lysate that had been made into 1 mmol/L concentration by mixing with PMSF 1.5 μl was then supplemented, and the total protein was extracted. The process was performed on ice at 4°C--centrifugal action took place at 10 000 r/min (with a semidiametre of 4 cm) for 5 min. After that, the supernatant was obtained to measure the protein concentration with BCA and denature at 99°C for 10 min. Subsequently, 50 μg protein was obtained for 10% SDSPAGE electrophoresis. The blots were electro transferred onto PVDF film and incubated in sealed suspension for 1 h. Following this, 1:100 diluted E-cad, vimentin, α-SMA primary antibody was added in to leave in overnight at 4°C. On the following day, the film was washed with TBST for 5 minutes and 3 times in a row. Lastly, 1:5000 HRP-marked secondary antibody and GAPDH were added in to incubate at 37°C for 2 h.

Statistical analysis

SPSS17.0 statistics software was employed to undergo statistical analysis. Both groups of measurement data had their difference compared using two sample t tests, whereas the pairwise comparison of rates utilized ×2 test or Fisher exact test. In addition, a two-tailed value of $P<0.05$ was considered statistically significant.

Results

Cell proliferation inhibition of DDP on breast cancer MCF-7 cells

The results of MTT results showed that DDP had a significant proliferation inhibitory effect on MCF-7 cells with a dose-dependent manner, the IC50 was 0.08 μmol/L ($P<0.05$, **Figure 1**).

TGF-β1 siRNA were transfected successfully

The Western blot results showed that compared with the control group, the expression of TGF-β1 protein in TGF-β1 siRNA group declined markedly ($P<0.05$, **Figure 2**). It was suggested that TGF-β1 siRNA successfully transfected into MCF-7 cells.

Changes of cell morphology after siRNA transfection

After 48 hours of cells culture, it was shown in the inverted microscope that the MCF-7 cells of the control group were in monolayer growth and cobblestone-like appearance. The cells were closely connected to each other (**Figure 3A**); the MCF-7 cells of DDP processing group were in spindle-shaped change and in disorder arrangement. The intercellular connection was loosened compared with the control group (**Figure 3B**); the MCF-7 cells of the DDP+TGF-β1 siRNA group further decreased with disordered arrangement. The intercellular connection was further loosened (**Figure 3C**).

Cell cloning inhibitory activity was enhanced after siRNA transfection

The result of single cell clone formation showed that the numbers of cell clone formation of three groups was (23.92±1.61), (8.59±1.38), and (5.43±0.56) respectively. DDP group and TGF-β1 siRNA group can significantly inhibit MCF-7 cell clone proliferation, but the numbers of cell clone of DDP+TGF-β1 siRNA group was significantly lower than that of DDP group and TGF-β1 siRNA group ($P<0.05$, **Figure 4**). It was showed that TGF-β1 siRNA can significantly enhance cell cloning inhibitory activity DDP to MCF-7 cells.

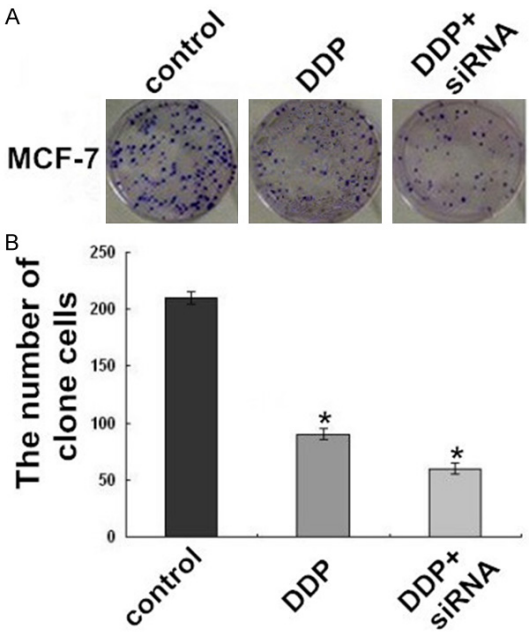

Figure 4. Effects of TGF-β1 siRNA on cell clone of MCF-7 cell lines.

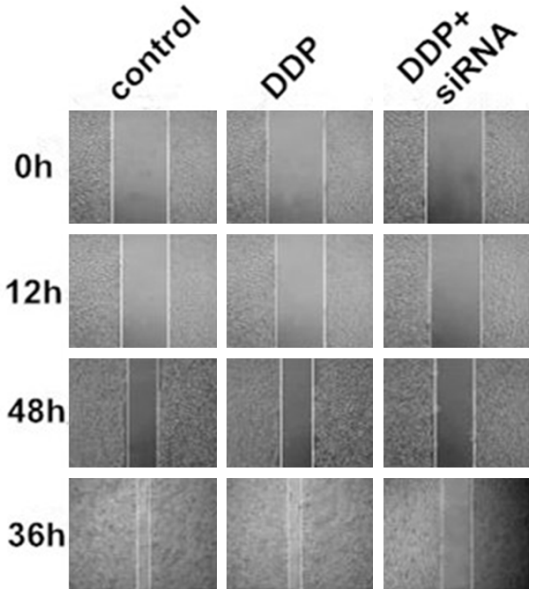

Figure 5. Effects of TGF-β1 siRNA on MCF-7 cell migration assessed by wound healing assay.

Cell migration inhibitory activity was enhanced after siRNA transfection

The cell wound scratch assay result showed that compared with the control group, DDP group and TGF-β1 siRNA group can inhibit the healing speed of MCF-7 cell wound scratch, but the latter had marked results (*P<0.05*, **Figure 5**). It was indicated that TGF-β1 siRNA can enhance cell migration inhibitory activity DDP to MCF-7 cells.

Cell invasion inhibitory activity was enhanced after siRNA transfection

Transwell Chamber Invasion results showed that compared with the control group, DDP group and TGF-β1 siRNA group can inhibit the invasion of MCF-7 cells, but the latter had marketed results (*P<0.05*, **Figure 6**). It was suggested that TGF-β1 siRNA can enhance cell invasion inhibitory activity DDP to MCF-7 cells.

TGF-β1 siRNA inhibit EMT

Western blot results showed that compared with the control group, down-regulated expression of Vimentin, mesenchymal cell marker, and α-SMA, extracellular matrix protein, was noted in DDP group and TGF-β1 siRNA group; but the down-regulated results was obvious in TGF-β1 siRNA group; in the meantime, compared with the control group, up-regulated expression of epithelial cell marker E-cadherin was shown in DDP Group and TGF-β1 siRNA group, but the latter had significant results (*P<0.05*, **Figure 7**). It was demonstrated that TGF-β1 siRNA can inhibit the occurrence of EMT.

Discussion

TGF-β signal can regulate the occurrence, development and metastasis of tumor through various mechanisms in tumor. The research shows that TGF-β has double-side effects during the process of tumor occurrence and development. It means that at the initial phase of tumor, TGF-β can inhibit the proliferation of tumor cells and induce the apoptosis of tumor cells. During the phase of tumor progression, excessive TGF-β will be generated through autocrine and paracrine of tumor cell and thus promote tumor invasion and metastasis. TGF-β mainly promotes tumor metastasis by ① regulating Smad protein to induce metastasis; ② regulating the effector that relies on the invasion and metastasis of Smad activation; ③ regulating the occurrence of tumor vessel; ④ inducing the changes of substrate environment surrounding the tumor [4-6]. The key singling molecule of TGF-β1 signal pathway is cytoplas-

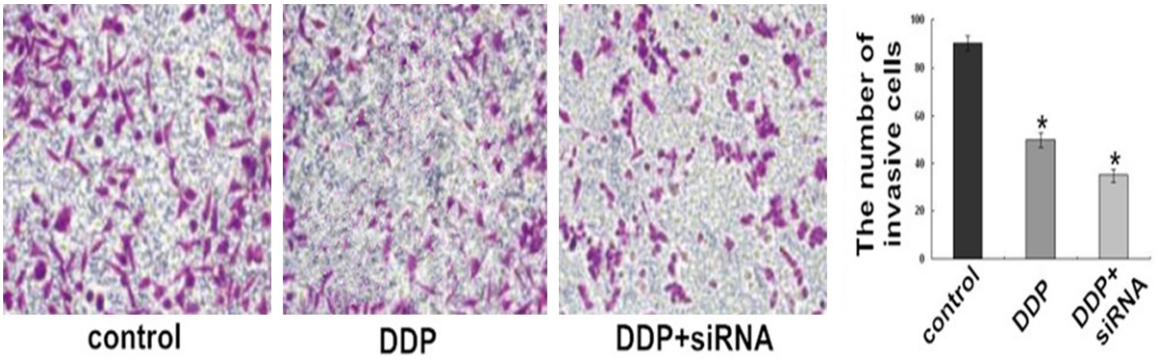

Figure 6. Effects of TGF-β1 siRNA on MCF-7 cell invasion assessed by Transwell chamber invasion.

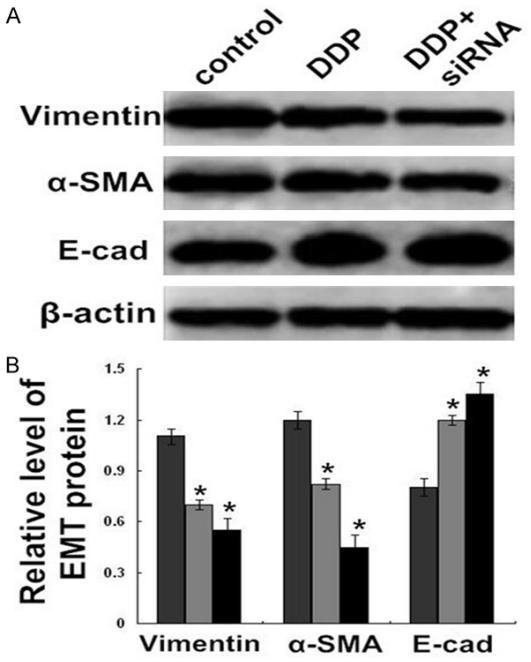

Figure 7. The expression levels of vimentin, α-SMA, and E-cad in MCF-7 cell lines detected by western blot. A: Protein blotting stripe. B: The relative level of vimentin, α-SMA, and E-cad protein. *P<0.05 versus the control group.

matic protein; TGF-β1 needs to combine with TGF-β1 receptor II, but the phosphorylation of receptor I phosphorylates Smad2/3 and combines with Smad4 to form tripolymer and transfer to cell nucleus, inhibit the epithelial factor and promote the expression of periplasmic protein. Smad4 is the unique mediated Smad in the signal transduction pathway, and plays an important role in the transmission process where TGF-β1 signal is transferred to the cell nucleus [7-9]. The correlation research on the invasion and migration activity of TGF-β1/Smad conduction path and cancer cell has also revealed that the down-regulation of Smad4 expression can block the transmission of this pathway and reduce the process of TGF-β1 signal to cell nucleus.

Epithelial-mesenchymal transition (EMT) is a process where epithelial cell loses polarity and obtains mesenchyme morphology [10-12]. In tumor cells, the EMT stimulation has accelerated the invasion and metastasis of tumor. EMT conversion is regarded as a precise regulation process, which can be divided into three aspects. 1. The epithelial cell loses adhesion characteristic [13, 14]. E-cadherin, as the classic epithelium molecular marker for trans-differentiation, plays an important role in cell integrity and polarity. It plays an important role in mediating intercellular adhesion. The decrease of E-cadherin has become a symbolic change during the EMT process; 2. The increase of expression of mesenchyme markers, such as Vimentin, FSP-1, etc; 3. The up-regulation of newly-generated extracellular matrix (Col4A1, FN and α-SMA). The research shows that TGF-β1/Smad signal path plays a key role in inducing EMT and promoting the invasion and metastasis of tumor [15-19].

The expression of siRNA and its combination with other drugs or classical pathway for tumor research have become a research focus in recent years, and it is of key significance to realize multi-path and multi-channel comprehensive treatment. On the basis of siRNA silence TGF-β1 gene, this study observes the influence and possible mechanism of DDP on inhibiting breast cancer MCF-7 cell proliferation, migration and invasion activation. After siRNA transfection, the cellular morphology has markedly

changed from cobblestone-like epithelial cell to spindle-shaped mesenchymal cell. Compared with the control group, both DDP group and TGF-β1 siRNA can inhibit MCF-7 cell proliferation, migration and invasion, but better effect is shown in TGF-β1 siRNA group. However, the western blot results show that compared with the control group, the down-regulation of Vimentin and α-SMA protein expression, as well as the up-regulation of E-cad expression is shown in DDP group and TGF-β1 siRNA group, but marketed tendency is particularly shown in TGF-β1 siRNA group.

In conclusion, TGF-β1 gene silencing enhance the sensitivity of breast cancer to cisplatin on proliferation, migration and invasion. Its function may partially associated with inhibition of the occurrence of EMT. In brief, TGF-β1 may become a new early effective diagnosis mark and potential therapy target in cisplatin sensitivity to breast cancer, it deserves further studying.

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Disclosure of conflict of interest

None.

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