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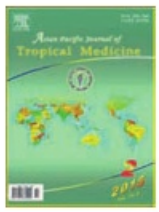

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Changes of TIZ expression in epithelial ovarian cancer cells

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ABSTRACT

Objective: To study the change of TIZ expression in epithelial ovarian cancer cells. **Methods:** HO8910 cells were transinfected with siRNA to inhibit the expression of TIZ. pcDNA3.1-TIZ vectors were combined to increase the TIZ expression level. The cell viability, colony forming efficiency and cycle distribution of HO8910, HO8910/NC, HO8910/pcDNA3.1-NC, HO8910/ TIZ-573 and HO8910/pcDNA3.1-TIZ were compared, and the invasion rate, migration rate and adhesion rate between 5 groups of cells were compared. **Results:** Compared with those of HO8910, HO8910/ NC and HO8910/pcDNA3.1-NC, the cell viability, colony forming efficiency and cell cycle distribution of HO8910/ TIZ-573 were increased, while the indexes of HO8910/pcDNA3.1-NC were decreased with statistical significant difference ($P<0.05$). There was no statistical significant difference in the invasion rate, migration rate and adhesion rate between 5 groups of cells ($P>0.05$). **Conclusions:** The expression of TIZ can inhibit the proliferation of epithelial ovarian cancer cells.

1. Introduction

The epithelial ovarian tumor is a common malignant gynecological tumor, occupying 50%-70% of ovarian tumors [1]. With the advances in the modern cellular and molecular biology, we have gained a deeper understanding in the occurrence and development of the epithelial ovarian cancer. Considerable research has indicated that such a tumor is the result of a combination of many genes and factors in different stages[2]. TRAF-6 inhibitory zinc finger protein (TIZ) belongs to the C_2H_2-type zinc finger protein family, which can be combined with the tumor necrosis factor receptor-associated factor 6 (TRAF-6) to play a role in the regulatio[3,4]. According to clinical research, the expression level of TIZ protein in the serum of patients with ovarian malignant tumor was significantly higher than the one of patients with ovarian benign tumor and the one of normal subjects, which indicates the high correlation between the expression level of TIZ and the ovarian cancer[5]. In this study, we observed the expression level of *TIZ* gene in epithelial ovarian tumor cells (HO8910) to study the effect of TIZ on the biological characteristics of tumor cells.

2. Materials and methods

2.1. Materials and reagents

The epithelial ovarian tumor cells (HO8910) provided by Shanghai SXBio Biotechnology Co., Ltd. was chosen as the experimental subject. pGPU6/GFP/Neo interference vector was provided by Shanghai GenePharma Co., Ltd. pcDNA3.1 expression vector, TRIzol, propidium iodide (PI), RPMI 1640 medium and liposome LipofectAMINE™2000 were all purchased from Invitrogen, a brand under the Life Technologies brand of the Thermo Fisher Scientific corporation. cDNA synthesis kit was provided by MBI Fermentas; DNA segment rapid purification recycle kit was provided by Beijing Sunbiotech Co., Ltd.; E.Z.N.A.® Plasmid Mini Kit I was provided by Omega Biotek. The fibronectin (FN), transwell chamber and artificial basement membrane matrigel were all provided by Coming Costar. *Escherichia coli* (*E. coli*) DH-5 α was preserved in this laboratory.

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2.2. Determination of TIZ expression level in HO8910 cell lines

TRIzol reagent was used to extract mRNA of HO8910 cells for the reverse transcription. cDNA amplification was performed with the reaction system of 25 μL. The reaction conditions were the degeneration at 94 ℃ for 5 min, at 94 ℃ for 1 min, at 5 ℃ for 1 min and at 72 ℃ for 1 min, 35 times of sequence cycles, extension at 72 ℃ for 5 min and the preservation of product at 4 ℃. The electrophoretic determination of PCR products was performed using 1% agarose gel. All the above primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

2.3. Interference of small RNA in TIZ expression

Three segments of small interfering RNA (siRNA) were designed and synthesized according to the mRNA sequences of TIZ in GenBank: the upstream segment of TIZ-554 was 5′-CACCUAACUCGACAUGAAATT-3′ and the downstream one was 5′-UUUCAUGUCGAGUUAGGUGTG-3′; the upstream segment of TIZ-573 was 5′-GAAAUUAUACCAAGGUGAATT-3′ and the downstream one was 5′-UUCACCUUGGUAUAAUUUCTT-3′; the upstream segment of TIZ-620 was 5′-GCUGUUAACCAAUCUUCAATT-3′ and the downstream one was 5′-UUGAAGAUIJCCImAACAGCTT-3′. Negative control (NC): the upstream segment was 5′-UUCUCCGAACGUCUCACGUTT-3′ and the downstream one was 5′-ACGUGACACGUUCGGAGAATT-3′. All the above sequences were synthesized by Shanghai GenePharma Co., Ltd.

TIZ-554, TIZ-573, TIZ-620 and negative control sequences were combined with T4-DNA ligase and pGPU6/GFP/Neo interference vectors respectively. E. coli DH-5 α competent cells were transformed by products respectively. The bacterial flora was screened using 100 μg/uL ampicillin and it was cultured for amplification. The plasmid was extracted and PCR and double restriction enzyme digestion were performed again. The positive recombinant plasmid was used for bi-directional sequencing of DNA.

HO8910 cells were transfected using the recombinant plasmids of TIZ-554, TIZ-573, TIZ-620 and NC, named as HO8910/TIZ-554, HO8910/TIZ-573, HO8910/TIZ-620 and HO8910/NC respectively. The above cells and untreated HO8910 cells were compared for the expression level of TIZ.

2.4. Upregulation of TIZ expression via in vitro transfection

PCR product and pcDNA3.1 vector were both treated by double digestion of *Bam*H Ⅰ and *Eco*R Ⅰ and the products of digestion were separated. Target fragments were recovered and purified. And the recombinant plasmid was constructed. T4-DNA ligase was used to combine the vector and the target gene. *E. coli* DH-5 α competent cells were transformed by the product (pcDNA3.1-TIZ). The extraction and detection of recombinant plasmid were the same as above. HO8910 cells were transfected using pcDNA3.1-TIZ plasmid and pcDNA3.1 plasmid, named as HO8910/pcDNA3.1-TIZ cell and HO8910/pcDNA3.1-NC cell respectively. mRNA of untreated HO8910 cells, HO8910/pcDNA3.1-TIZ cells and HO8910/pcDNA3.1-NC cells was extracted respectively and RT-PCR was used to detect the expression level of TIZ in cells.

2.5. Detection of cell viability by MTT method

Cells in the logarithmic phase were taken to prepare the single cell suspension, with a cell density of 1×10^5/mL. A total of 100 μL of cell suspension were added in the 24-well plate, with 3 repeated wells. The control group was set with 100 μL of medium in each well. A total of 100 μL of 5 mg/mL MTT solution was added in each well and incubated at 37 ℃ for 4 h. The liquid was removed, 500 μL DMSO was added in each well and oscillated for 10 min. Absorbance (A) value was measured at 450 nm and the growth curve of cells was drawn.

2.6. Colony formation assay

Stably transfected cells were chosen to prepare the single cell suspension. Cells were seeded in the 24-well plate, with 100 cells in each well. The 24-well plate was gently shaken to achieve the uniform distribution of cells. Cells were put in the thermotank of 37℃ with 5% CO_2 volume fraction for the culture of 5-7 days. After the culture, cells were washed using PBS. Cells were stained by Giemsa. The number of colony forming units was counted under the optical microscope. A total of 15-50 cells were taken as a colony. The equation was as follows: Colony-forming efficiency (%) = Number of clones / Number of inoculated cells × 100%.

2.7. Determination of changes in cell cycle using flow cytometry

Cells were taken in the logarithmic phase to prepare the single cell suspension. A total of 15 mL of 95% ethanol solution pre-cooled at -20 ℃ was added and fixed at 4 ℃ for 1 h. The ethanol solution was removed using the centrifugal method. The cell density was adjusted to 1×10^6/mL, digested using 50 μg/mL RNA enzyme for 2 min and strained by using 50 μg/mL PI away from light for 30 min. The content distribution of DNA was analyzed by using flow cytometry, as well as the proportion of cells in phases of G_0/G_1, G_2+M and S. The proportion of cells in the phase of G_0/G_1 was taken as the indicator to reflect the proliferation ability of cells.

2.8. Changes in in vitro invasion and migration

Polycarbonate membrane filter of Transwell was treated using PBS with 5 μg FN. A total of 50 μL of 1.25mg/mL matrigel was added in the upper cavity. The treated

Transwell was incubated at 37 ℃ for 4–5 h. The cell suspension was added in the upper and lower cavity. After 24 h, MTT method was used to detect D value of adhesion cells in upper and lower cavities at 450 nm. The equation was as follows:Invasion rate (%) = D_{450nm} of Cells in lower cavity/D_{450nm} of cells in upper cavity × 100% [5].

Except the addition of matrigel, the determination of in vitro migration was similar with the in vitro invasion assay. Migration rate (%) = D_{450nm} of cells in lower cavity/D_{450nm} of cells in upper cavity × 100%[5].

2.9. Changes in in vitro adhesion ability

The 96-well plate was taken, and 50 μL of 20 mg/L FN was added in each well, allowing it to air dry on the clean bench over the night, and then the plate was put in the refrigerator at 4 ℃. Before using, it was used by PBS twice. The binding site was closed, the cell suspension was added, cultured for 1 h and washed by PBS to remove the unadhered cells. MTT method was used to detect D value of adhesion cells in upper and lower cavities at 450 nm. Adhesion rate (%) = D_{450nm} of adhesion cells in experiment group/D_{450nm} of adhesion cells in control group × 100%[6]. Repeat the procedures (2.4–2.8) for three times.

2.10. Statistical analysis

Statistical analysis was performed by using SPSS15.0. The measurement data were expressed as mean±sd. The one-way ANOVA was used to analyze the difference between groups, while the analysis of variance with repeated measures was used in comparison of cell viability at different times, $P<0.05$ indicating the statistical significant difference.

3. Results

3.1. Determination of siRNA interference efficiency

Compared with NC sequence transfected cells, the expression level of TIZ in three siRNA transfected fragments of TIZ-554, TIZ-573 and TIZ-620 was significantly decreased ($P<0.05$); the expression level of TIZ in TIZ-573 transfected cells was significantly lower than others ($P<0.05$). There was no statistical difference in the expression level of TIZ between HO8910/NC and HO8910 ($P>0.05$), as shown in Figure 1.

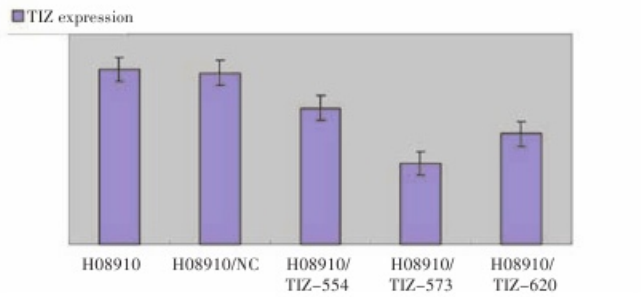

Figure 1. Expression level of TIZ in different cells.

3.2. TIZ expression in pcDNA3.1-TIZ transfected cells

There was no statistical difference in the TIZ expression between HO8910 and HO8910/pcDNA3.1-NC ($P>0.05$), but TIZ expression in HO8910/pcDNA3.1-TIZ cells was significantly increased ($P<0.05$), as shown in Figure 2.

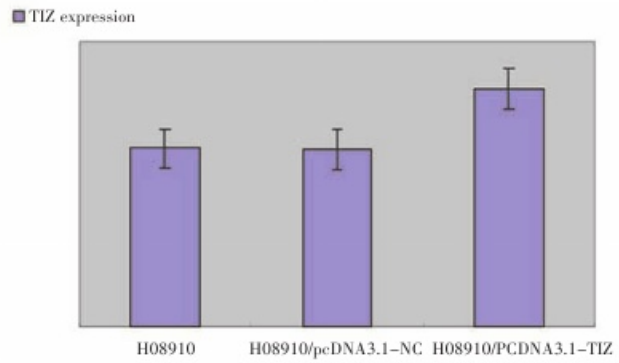

Figure 2. Expression level of TIZ in different cells.

3.3. Effect of TIZ expression on cell viability

TIZ-573siRNA fragments with the best interference effect were chosen for the study. There was no statistical difference in the cell viability between HO8910, HO8910/NC and HO8910/pcDNA3.1-NC ($P>0.05$). The cell viability of HO8910/ TIZ-573 was significantky increased, while the one of HO8910/pcDNA3.1-TIZ was significantly decreased ($P<0.05$), as shown in Figure 3.

3.4. Effect of TIZ expression on colony forming efficiency

There was no statistical difference in the colony forming efficiency between HO8910, HO8910/NC and HO8910/

Table 1
Colony forming efficiency of different cells (mean±sd).

Cell	Number of seeded cells	Colony forming efficiency (%)
HO8910	100	59.23±4.15
HO8910/NC	100	59.11±4.22
HO8910/pcDNA3.1-NC	100	58.88±3.97
HO8910/ TIZ-573	100	84.12±7.45
HO8910/pcDNA3.1-TIZ	100	41.55±2.43

pcDNA3.1-NC (P>0.05). The colony forming efficiency of HO8910/ TIZ-573 cell was increased, while the one of HO8910/pcDNA3.1-TIZ cell was decreased, with the statistical difference (P<0.05), as shown in Table 1.

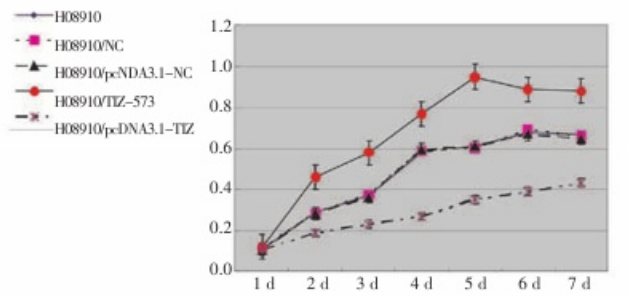

Figure 3. Growth curves of different cells.

3.5. Effect of TIZ expression on cell cycle

There was no statistical difference in the percentage of cells in different phases between HO8910, HO8910/NC and HO8910/pcDNA3.1-NC (P>0.05). Cells of HO8910/ TIZ-573 in the phase of G_0/G_1 were significantly increased, while cells of HO8910/pcDNA3.1-TIZ in the phase of G_0/G_1 were significantly decreased (P<0.05), as shown in Table 2.

3.6. Effect of TIZ expression on in vitro invasion, migration and adhesion of cells

There was no statistical difference in the *in vitro* invasion rate, migration rate and adhesion rate between HO8910, HO8910/NC, HO8910/pcDNA3.1-NC, HO8910/ TIZ-573 and HO8910/pcDNA3.1-TIZ (P>0.05), as shown in Table 3.

4. Discussion

According to the clinical studies, the expression level of TIZ protein in the serum of patients with ovarian malignant tumor was significantly higher than the one of patients with ovarian benign tumor and the one of normal subjects, which indicates the high correlation between the expression level of TIZ and the ovarian cancer. In this study, we used siRNA to interfere with the expression level of TIZ in HO8910 and then constructed the expression vector to increase the expression level of TIZ. Relying on the *in vitro* experiment, we studied the effect of change in the expression level of TIZ on the biological characteristics of epithelial ovarian tumor cells (HO8910).

In the study, compared with that in transfected cells of NC sequence, the TIZ expression in three segments of cells transfected by siRNA, namely TIZ-554, TIZ-573 and TIZ-620, was significantly decreased, while the transfection efficiency of TIZ-573 was the highest; the TIZ expression in HO8910/pcDNA3.1-TIZ cell was significantly increased, which indicates the success of interference in the TIZ expression in HO8910.

TIZ-573siRNA segment with the best interference effect was selected for the further study. The cell viability, colony forming efficiency and cell cycle of HO8910, HO8910/NC, HO8910/pcDNA3.1-NC, HO8910/ TIZ-573 and HO8910/ pcDNA3.1-TIZ were compared and then the proliferation ability of different cells was evaluated. Compared with that of HO8910, HO8910/NC and HO8910/pcDNA3.1-NC, the cell viability of HO8910/ TIZ-573 was increased, as well as the colony forming efficiency and the number of cells in the phase of G_0/G_1; while the cell viability of HO8910/pcDNA3.1-TIZ was decreased, as well as the colony forming efficiency and the number of cells in the phase of G_0/G_1. It indicates that the expression of TIZ inhibits the proliferation of epithelial ovarian cancer cells. TIZ is just like the hospital of zinc finger protein KRAB family. Such kind of protein all plays a critical role in the embryonic development, transformation and differentiation of cells and the regulation of cell cycles[7,8]. The mechanism for TIZ to inhibit the proliferation of tumor cells has not been clear, but some research has indicated that it can effectively inhibit the TRAF-6 mediated signal transduction. It combines with N terminal of TRAF-6 to change the protein conformation of TRAF-6 and then blocks the transduction of downstream signals[9]. The TRAF-6 mediated signal transduction is

Table 2
Cell cycle of different cells (mean±sd, %).

Cell	G_0/G_1	G_2+M	S
HO8910	47.22±5.18	31.66±3.55	24.58±2.17
HO8910/NC	48.12±5.01	27.98±3.14	25.55±2.09
HO8910/pcDNA3.1-NC	48.85±5.52	29.13±3.35	23.82±2.23
HO8910/ TIZ-573	35.67±3.38	24.45±2.35	41.24±5.64
HO8910/pcDNA3.1-TIZ	65.88±7.02	17.54±1.97	18.65±1.88

Table 3
In vitro invasion, migration and adhesion of different cells (mean±sd, %).

Cell	Invasion rate	Migration rate	Adhesion rate
HO8910	45.25±4.87	53.16±5.13	64.62±7.65
HO8910/NC	45.82±4.47	55.42±5.67	65.53±7.43
HO8910/pcDNA3.1-NC	44.72±4.85	54.76±5.44	63.88±6.92
HO8910/TIZ-573	47.71±5.02	56.82±5.65	67.03±8.22
HO8910/pcDNA3.1-TIZ	44.12±4.28	52.97±5.21	63.37±7.14

relatively complicated, mainly including two ways[10]: (1) TRAF-6→mitogen-activated protein kinase kinase (MAPKK) →mitogen-activated protein kinase (MAPK); (2)TRAF-6 →MAPKK→nuclear factor KB (NF-κB). The downstream signal proteins involved in the above two ways, such as JNK, P38, ERK1/2, C-FOS and NF-κB, are all closely related to the occurrence and development of ovarian cancer[11,12]. By blocking the TRAF-6 mediated signal transduction, TIZ can significantly inhibit the expression of the above proteins and thus inhibit the proliferation of epithelial ovarian cancer cells (HO8910).

In this study, there was no statistical difference in the invasion rate, migration rate and adhesion rate between HO8910, HO8910/NC, HO8910/pcDNA3.1-NC, HO8910/TIZ-573 and HO8910/pcDNA3.1-TIZ, which indicates that there is no significant effect of TIZ expression on the *in vitro* invasion, migration and adhesion of epithelial ovarian cancer cells. The invasion ability of ovarian malignant tumor was relatively strong. 76% diagnosed patients had a wide migration of the abdominal cavity, the pelvic plant and the liver substance or the pleura and the brain[13]. The migration is the result of combination of cell adhesion and new blood vessels. Presently, there has been no definite conclusion on the correlation between TIZ expression and the spread and migration of epithelial ovarian tumor. Some research indicated that in the TIZ-mediated signal transduction, there was a high correlation between the cell factors of lysophosphatidic acid, matrix metalloproteinases, urokinase-type plasminogen activator, vascular endothelial growth factor and intercellular adhesion molecule and the invasion and migration of ovarian cancer[14,15]. The downstream signal molecules of TIZ expression had a negative feedback to the expression of TIZ, which then decreased the expression level of TIZ and weakened the inhibition ability; the decrease in the expression level of TIZ would also inhibit the activation of downstream signaling pathways to deactivate the signal molecules related to the invasion and migration of tumor and then decrease its invasion ability[16,17]. Anyway, TIZ possesses the dual-direction regulation on the invasion ability of HO8910. In conclusion, this study indicates that TIZ expression can inhibit the proliferation of epithelial ovarian tumor, but has no obvious effect on the invasion ability of tumor.

Conflict of interests

We declare that we have no conflict of interest.

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