ORIGINAL ARTICLES

Impacts of silencing CD105 and Ki67 gene expression on the biological behavior of ovarian cancer OVCAR3 cells

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Abstract

Objective: To investigate the effect of silencing CD105 and Ki67 gene expression on OVCAR3 cell line in ovarian cancer.
Methods: According to cell transfection results, OVCAR3 cells were divided into CD105-siRNA, Ki67-siRNA, CD105-siRNA + Ki67-siRNA, negative control (NC1 and NC2) and control (non-transfection) groups. The effect of silencing CD105, Ki67 and their combined gene on proliferation, migration, invasion and apoptosis of human ovarian cancer cells can be studied and analyzed with MTT assay, wound-healing assay, Transwell chamber and flow cytometry.
Results: Compared with their respective negative control and liposome control groups, proliferation, migration and invasion abilities of OVCAR3 cells were decreased obviously in CD105-siRNA, Ki67-siRNA and CD105-siRNA + Ki67-siRNA groups after gene silencing. Whereas, the most significant change was detected in CD105-siRNA + Ki67-siRNA group along with obviously increased apoptosis rate of cancer cells, and the differences were of statistical significance ($p < .01$, $p < .05$).
Conclusions: Expression vectors of CD105-siRNA and Ki67-siRNA can inhibit the proliferation of OVCAR3 cells, reduce their abilities of migration and invasion, and induce apoptosis of tumor cells. The effect of combined gene silence will be more significant.

Key Words: Ovarian cancer, CD105, Ki67, Proliferation, Migration, Western blotting, Human

The occurrence of ovarian cancer is a multi-factor, multi-step, multi-stage interaction process involving a series of genetic changes, not only including cell hyperplasia caused by oncogene activation and/or anti-oncogene inactivation associated with cell proliferation and differentiation, but also tumor metastasis caused by gene imbalance related to cell invasion and migration.

CD105 (Endoglin) is a good marker of angiogenesis, and there is some evidence that CD105 can regulate not only cell proliferation and aggregation, but also migration of tumor cells;[1,2] cell proliferation-associated nuclear antigen Ki67 (Ki67) is also an excellent indicator reflecting the proliferation of ovarian cancer cells.[3] They both play an important role in the development, treatment and general prognosis of tumors.

OVCAR3 cell line cultured in vitro was applied to the experiment. The conditions of proliferation, migration, invasion and apoptosis of tumor cells after interference (by inhibiting the expression of CD105 and Ki67) can be studied and analyzed with MTT assay, wound-healing assay, Transwell chamber and flow cytometry, in order to observe the effect of CD105 and Ki67 on the biological behavior of ovarian cancer cells.

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1 Materials and methods

1.1 Main reagents

Human epithelial ovarian cancer OVCAR3 cell line was purchased from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Beijing Aoke Dingsheng Biotech Co., Ltd. was commissioned to synthesize small interfering RNA (siRNA).

1.2 Experimental methods

1.2.1 Cell culture, transfection and grouping

OVCAR3, SKOV3TRp2, HIO-180, ES2, HeyA8MDR and A2780ip2 cells were placed in DMEM medium (containing 10% FBS) after cell resuscitation. Until cells reached a relatively stable state, the expression and the location of CD105 and Ki67 in 6 strains of cells were detected by Western blotting. When OVCAR3 cells with the highest expression of CD105 and Ki67 were cultured until approaching about 90% confluence, CD105-siRNA, Ki67-siRNA, CD105-siRNA + Ki67-siRNA and their respective control siRNA were transfected into OVCAR3 cells, with a final concentration of 50 nmol/L. After transfection, the cells were divided into 6 groups for experiments: control group, negative control groups (for control use of siRNA, including NC1 and NC2), CD105-siRNA group (transfection with CD105-siRNA), Ki67-siRNA group (transfection with Ki67-siRNA), CD105-siRNA + Ki67-siRNA group (transfection with Ki67-siRNA and CD105-siRNA).

1.2.2 The change in the proliferation ability of OVCAR3 cells after interference detected with MTT assay

Experiment grouping: A. background: complete medium + MTT, no cells; B. control group: complete medium + MTT + OVCAR3 cells; C. negative control groups: complete medium + MTT + OVCAR3 cells + transfected negative siRNA for control use; D. experimental group: complete medium + MTT + OVCAR3 cells + transfected CD105-siRNA.

Cells in the logarithmic phase were collected with about 100 μl/well (around 1×10^4), and incubated. After 24 h, 48 h and 72 h, the cell culture was terminated respectively, with MTT reduced to formazan. It was required to dissolve formazan. The absorbance (A) value of each well was measured by the microplate reader (490 nm). When colorimetric assay was used, the blank well was with zero setting to calculate the inhibition rate of cell proliferation. Inhibition rate (%) = (A value of the control group - A value of the interference group)/A value of the control group $\times$ 100%.

1.2.3 The change in the migration ability of OVCAR3 cells after different means of interference detected with wound-healing assay

OVCAR3 cells were cultured. By use of a sterile pipette (10 μl), each well was marked with a "horizontal-line" styled scratch along the bottom of the culture plate. The relative distance of the scratch area was recorded under the microscope. Rinsed gently with serum-free medium twice to remove the cells that had been scratched out. The medium was replaced with RPMI1640 medium containing 10 g/bovine serum albumin (BSA) and 1% FBS (V/V) for another 24-hour incubation. Again, the medium was replaced with RPMI-1640 medium containing 10% FBS (V/V). The cells were cultured for another 24 hours and photomicrographed under an inverted phase contrast microscope. The scratch width (0 h) was measured under the condition of Image J 1.43 u. Then the cells were placed in an incubator (37°C, 5% CO_2) for continuous incubation. 16 h and 24 h later, the cells were photomicrographed with the same method mentioned above to measure the scratch width.

1.2.4 The change in the invasion ability of OVCAR3 cells after different means of interference detected with Transwell chamber

Collected OVCAR3 cells untreated as well as the same cells treated by CD105-siRNA + Ki67-siRNA, CD105-siRNA, Ki67-siRNA and NC-siRNA respectively. The cells were placed in the incubator (37°C) for 48 hours. The cells were fixed with 950 ml/L of ethanol for 15-20 min and stained with trypan blue for 5 min. The number of cells that passed through the membrane was observed under the inverted microscope. Cells in five randomly chosen fields were counted, and the invasion ability of the tumor cells was represented by the relative number of invasive cells. Removed the Transwell chamber, and washed off non-adherent cells. Those cells were fixed with 4% paraformaldehyde for 30 min, and stained with crystal violet for 10-30 min. Took 9 fixed fields represented by black dots in the figure (see Figure 5). Then, the cells were observed, photomicrographed and counted under the microscope. The results were analyzed by *t*-test.

1.2.5 The apoptosis of OVCAR3 cells after interference with CD105 and Ki67siRNA detected with flow cytometry

Collected and dissociated cells, which were resuspended gently with PBS after centrifugation and counted. Took 1 $\times 10^6$ resuspended cells, and the supernatant was discarded after centrifuging cells. 195 μl of Annexin V-FITC binding buffer was added to gently resuspend the cells. The cells were incubated in a light-proof place at room temperature

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(20-25°C) for 10 min. After centrifugation, the supernatant was discarded. 190 μl of Annexin V-FITC binding buffer was added to gently resuspend the cells. Added 10 μl of propidium iodide (PI) staining solution and mixed gently, then placed them in ice bath under the light-proof condition. It was required to detect the cells with flow cytometry immediately. Annexin V-FITC indicated green fluorescence, and PI showed red fluorescence. In the scatter diagram presented by the bivariate flow cytometer, the majority of the left lower quadrant showed living cells (FITC-/PI-); cells in the upper right quadrant were almost cells in the late apoptosis (FITC+/PI+); and most cells in the lower right quadrant were in the early apoptosis (FITC+/PI-). The software applied to data analysis is Cell Quest Software (Becton-Dickinson). The sum of the cells in the early and the late apoptosis stages was adopted to calculate the apoptosis rate.

1.2.6 Statistical analysis

SPSS 13.0 statistical software was applied to data analysis. The experimental data was represented by mean ± standard deviation ($\bar{x}$ ± s) with the application of two-sample group *t*-test. The difference $p < .05$ was of statistical significance.

2 Results

2.1 Changes in the proliferation ability of cells

The results of MTT assay showed that the cell growth rates were lower slightly both in the single interference and the combined interference groups at the time point of 24 h. Compared with liposome control groups, the inhibition rates of cell proliferation in CD105 group, Ki67 group and CD105 + Ki67 group were 19.72%, 22.79% and 41.33% respectively. At the time point of 48 h, the inhibition effect in each group was more significant, with the rates of 25.15%, 29.31% and 41.1% respectively. The inhibition rates were slightly higher at the time point of 72 h, reaching 29.15%, 33.65% and 55.4% respectively, and the difference ($p < .05$) was of statistical significance. While, the inhibition of cell proliferation in their respective negative control groups were not obvious from 24 h to 72 h (inhibition rates: 3.87%, 6.4%, 2.48%) and (2.18%, 4%, 0.8%). Compared with liposome control groups, the difference ($p > .05$) was of no statistical significance. The data showed that the inhibition of proliferation had already appeared from the 24-hour and reached the peak at the time point of 48 h. The inhibition still existed until the 72-hour, but it was less significant than that at the time point of 48 h (see Table 1 and Figure 1).

Table 1: Absorbance of OVCAR3 cells after transfection (detected with MTT assay) (n = 5, $\bar{x}$ ± s)

Group	24 h	48 h	72 h
CD105-siRNA	0.324 ±0.02	0.4422 ±0.05	0.6404 ±0.05
Ki67-siRNA	0.3116 ±0.01	0.4176 ±0.05	0.5998 ±0.04
CD105-siRNA + Ki67-siRNA	0.2364 ±0.03	0.348 ±0.03	0.4032 ±0.03
NC1 (CD105 blank control group)	0.388 ±0.05	0.553 ±0.07	0.8816 ±0.05
NC2 (Ki67 blank control group)	0.3948 ±0.01	0.5672 ±0.03	0.898 ±0.06
Control	0.4036 ±0.08	0.5908 ±0.03	0.904 ±0.04

Note. Compared with the 3 control groups, the difference was of statistical significance, $p < .05$

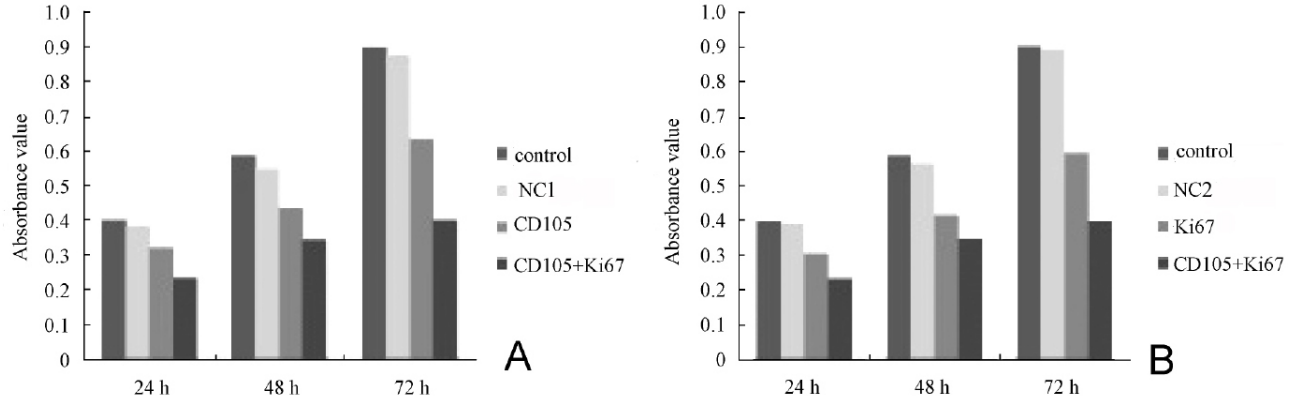

Figure 1: Proliferation ability of OVCAR3 cells after transfection (detected with MTT assay)

2.2 The change in the migration ability of cells after interference detected with wound-healing assay

After transfection with CD105-siRNA or Ki67-siRNA at a final concentration of 50 nmol/L, the cells were placed under the microscope again 48 h later to capture images and measure the scratch width after cells migrated to the scratch area. The experimental results showed that the migration distances in CD105 group, Ki67 group and CD105 + Ki67 group were significantly longer than those in liposome control groups, and the difference ($p < .05$) was of statistical significance. Nevertheless, the migration distance in each negative control group (NC1, NC2) showed no obvious difference from that in liposome control groups ($p > .05$, see Figures 2-4).

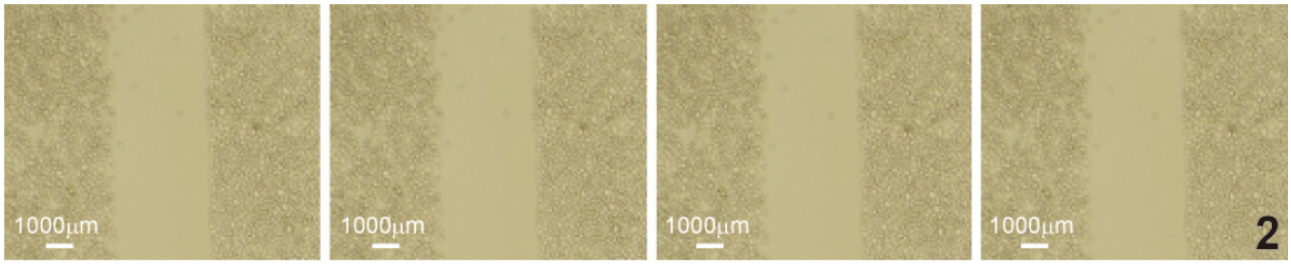

Figure 2: OVCAR3 cells before transfection (control, bar = 1000 μm)

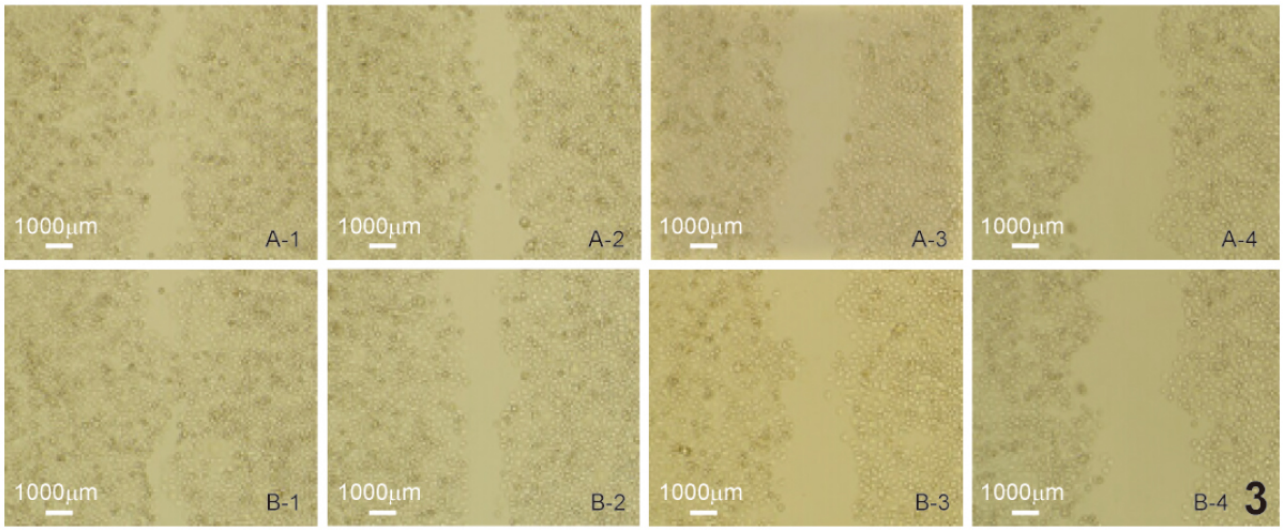

Figure 3: OVCAR3 cells at 48 h after transfection (detected with wound-healing assay, bar = 1000 μm)
The migration distances in CD105-siRNA group, Ki67-siRNA group and CD105 + Ki67 group were significantly longer than those in liposome control groups, and the difference ($p < .05$) was of statistical significance. Nevertheless, the migration distance in each negative control group showed no obvious difference from that in liposome control group ($p > .05$). A-1. CD105 blank control group; A-2. CD105 liposome control group; A-3. CD105-siRNA; B-1. Ki67 blank control group; B-2. Ki67 liposome control group; B-3. Ki67-siRNA; Combined interference group: A-4 and B-4; Negative control group: A-1 and B-1.

2.3 The change in the invasion ability of OVCAR3 cells in each group after transfection detected with Transwell chamber

After transfection with CD105-siRNA or Ki67-siRNA at the final concentration of 50 nmol/L, under the condition of HPF (× 200), five fields of cells were chosen randomly from each group, with the number of transmembrane cells counted. The counting results were as follows: CD105-siRNA group: 26.8 ± 2.3; Ki67-siRNA group: 32.2 ± 3.9; NC1: 45.2 ± 4.3; NC2: 50 ± 3.1; control group: 50.2 ± 2.5. Compared with negative control groups and liposome control groups, the number of transmembrane cells was decreased significantly ($p < .01$); it was more obvious that the average number of transmembrane cells was decreased in the combined interference group (11.8 ± 3.8, $p < .01$, $p < .05$). There was no significant difference in the number of transmembrane cells between negative control groups and the control group ($p > .05$, see Table 2 and Figure 5).

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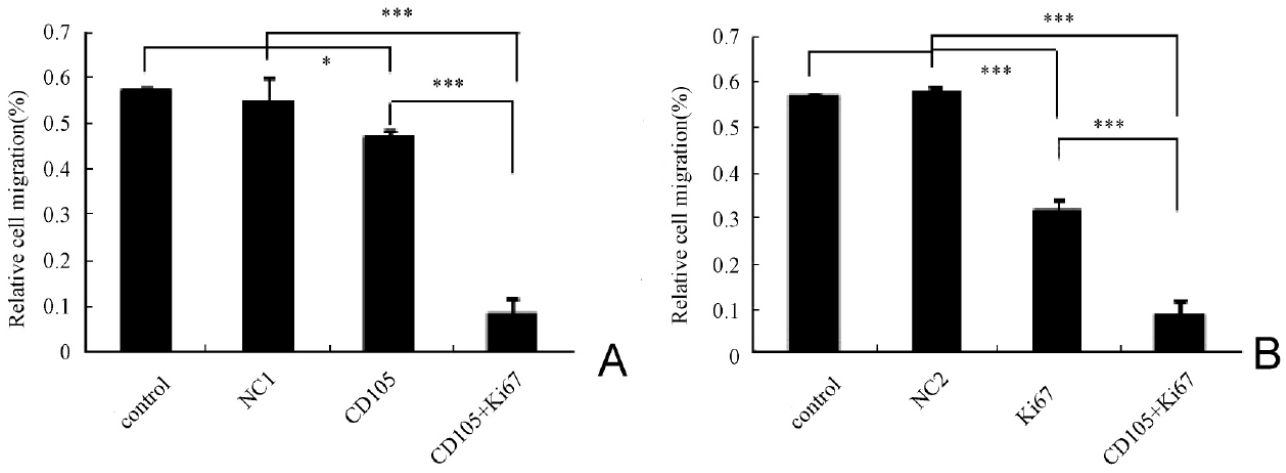

Figure 4: Migration ability of cells to the scratch area before and after transfection (detected with Wound-Healing assay, * $p < .05$; *** $p < .001$)

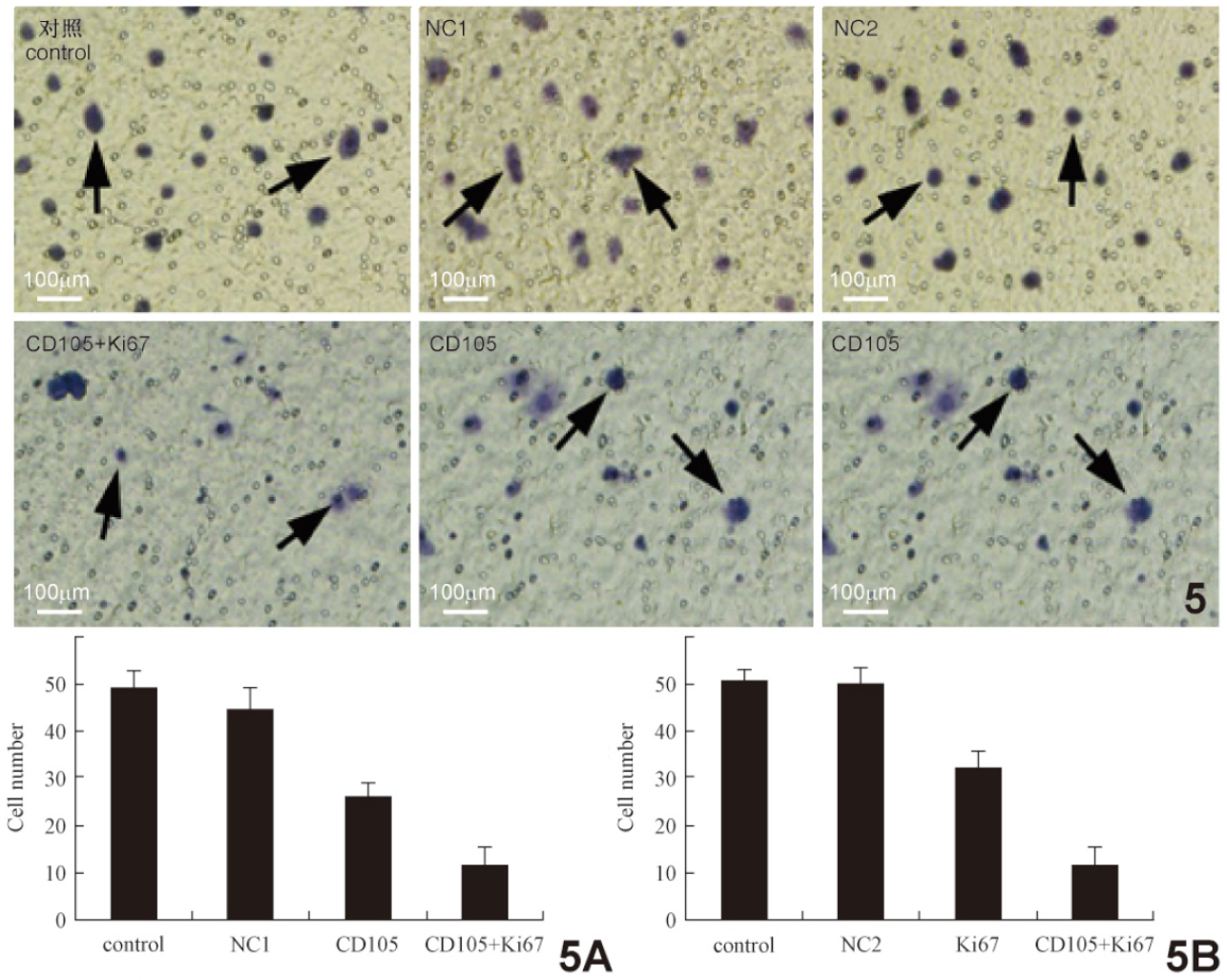

Figure 5: Invasion ability of OVCAR3 cells after transfection with siRNA (detected with Transwell assay, bar = 100 μm)

Table 2: Comparison of the number of transmembrane cells among groups after transfection (n = 5, $\bar{x}$ ± s)

Group	Cell number
CD105-siRNA	26.8 ±2.3
Ki67-siRNA	32.2 ±3.9
CD105-siRNA + Ki67-siRNA	11.8 ±3.8
NC1 (CD105 blank control group)	45.2 ±4.3
NC2 (Ki67 blank control group)	50 ±3.1
Liposome control group	50.2 ±2.5

2.4 Changes in the apoptosis of OVCAR3 cells in each group after transfection

In order to further understand the mechanism of silencing CD105 and Ki67 genes to the changes in the proliferation ability of ovarian cancer OVCAR3 cells, we also used flow cytometer to make an apoptosis analysis of cells in 4 groups (liposome control group, negative control group, single interference group and combined interference group). The results showed that the apoptosis rate after combined interference was (56.77 ± 3.58)%, 2.21 times higher than that in CD105 group (25.63 ± 1.76)%, 1.82 times higher than that in Ki67 group (31.23 ± 3.20)%, 11.27 times higher than that in the blank control group (5.03 ± 0.98)% and 17.74 times higher than that in the negative control group (3.2 ± 1.21)%. See Figure 6 and Figure 7 for details. It can be considered that the expression of both CD105 and Ki67 genes has an inhibitory effect on the apoptosis of ovarian cancer cells.

3 Discussion

CD105 (Enderlin) is a receptor protein that can bind to transforming growth factor-β (TGF-β) on vascular endothelial cells. Enderlin has been found gradually by scholars on epithelial cells in normal tissues or in vitro. Subsequently, the monoclonal antibody that identifies Enderlin in different laboratory is numbered as CD105.[4,5] CD105 is a disulfide-bond-linked homodimer membrane-bound glycoprotein with a relative molecular mass of 180 kD. Consisting of 633 amino acids, this glycoprotein is one of components in TGF-β complex. CD105, a specific marker of neovascular endothelial cells in tumor tissues, is related to tumor angiogenesis, which plays an important role in the development of malignant tumors. CD105 has a high expression in vascular endothelial cells of malignant tumors, including ovarian cancer, leukemia, gastrointestinal stromal tumors (GISTs), melanomas and laryngeal cancer. However, it is rarely expressed in non-endothelial cells.[6,7] At present, scholars at home and abroad mainly focused on the role of CD105 as an angiogenic factor and a marker for microvessel density in tumors, to determine the growth, prognosis and treatment effect of tumors.[7]

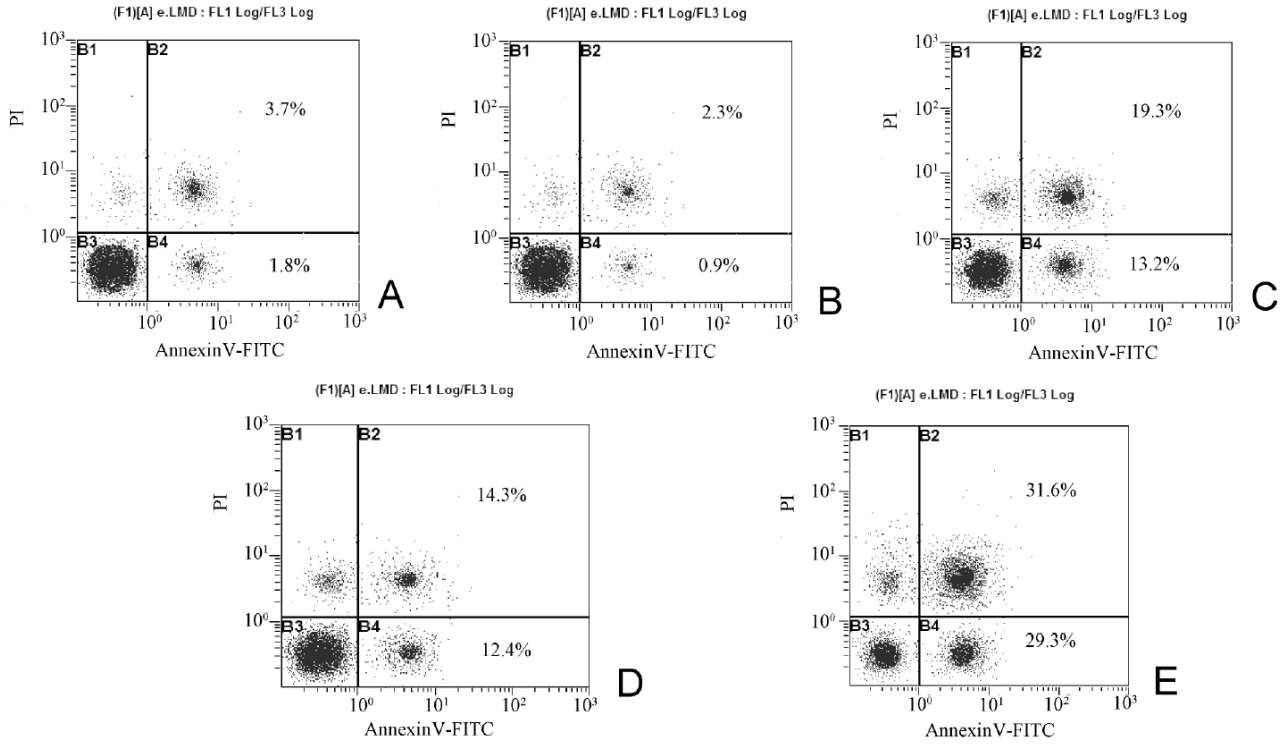

Figure 6: Apoptosis of OVCAR3 cells after silencing CD105 and Ki67 (detected with the flow cytometer)
A. Blank control group; B. NC1 and NC2; C. CD105-siRNA; D. Ki67-siRNA; E. CD105-siRNA + Ki67-siRNA

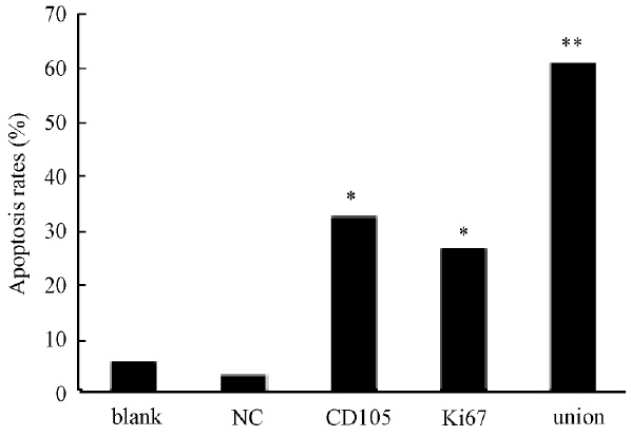

Figure 7: The change in apoptosis of OVCAR3 cells after silencing CD105 and Ki67

Proliferation-associated nuclear antigen Ki67 (Ki67), a type of protein that related to cell cycle, is the most representatively proliferative indicator among known proliferating antigens. Ki67 is mainly used to evaluate biological behaviors of cell proliferation activity, cell cycle and tumor growth, infiltration, recurrence and metastasis, i.e., to determine tumor proliferation activity and malignancy. The expression of Ki67 in ovarian cancer is significantly higher, which is closely related to the occurrence and development of ovarian cancer.[8–10] The effect of Ki67 expression on tumor prognosis and its relationship with the clinicopathological features of tumors suggest that Ki67 plays an important role in many different kinds of cancers.[11, 12] Therefore, Ki67 is a relatively reliable marker of cell proliferation. Previous studies have shown that the Ki67 proliferation index is associated with bad and long-term disease-free survival and overall survival of some cancers.[11–13]

This experimental study showed that 50 nmol/L of CD105-siRNA and ki67-siRNA expression vectors were re-transfected into human ovarian epithelial carcinoma OVCAR3 cells with high expression of CD105 and Ki67 in vitro. And then, proliferation, migration, invasion and apoptosis of human ovarian epithelial carcinoma OVCAR3 cells under the condition of silencing CD105, Ki67 and both CD105 and Ki67 were studied and analyzed respectively. The results showed that abilities of proliferation, migration and invasion of human ovarian epithelial carcinoma OVCAR3 cells were obviously decreased after silencing CD105 and Ki67 genes respectively. It was more significantly decreased after silencing both. It is considered that CD105 and Ki67 genes can inhibit the apoptosis of human ovarian epithelial carcinoma OVCAR3 cells, and the combination of the two genes can inhibit the apoptosis more obviously. It has been reported in the literature[14] that both CD105 and Ki67 are strongly expressed in endothelial cells with active tumor proliferation and are uniform in expressing the proliferation status of tumor cells. Their strong expression can prompt the proliferation activity of tumor cells. Our experimental results can provide a strong evidence for this view. Combined detection of CD105 and Ki67 in clinical practice may be of great help to determine the malignancy and prognosis of diseases.

In conclusion, the topic is devoted to analyzing the expression of CD105 and Ki67 in ovarian epithelial cancer cells and silencing single gene or combined genes specifically by using RNAi technology, to intervene and detect the expression of these two factors and observe the changes in proliferation, migration, invasion and apoptosis of ovarian cancer cells. This study is also intended to explore how CD105 and Ki67 as an independent factor or a combined factor transduce signals in occurrence and development of ovarian epithelial cancer, and determine the effect of combined detection of CD105 and Ki67 proteins on biological behaviors of ovarian epithelial cancer OVCAR3 cells. It provides the future targeted therapy of ovarian cancer with a laboratory evidence.

Conflicts of Interest Disclosure

The authors have no conflicts of interest related to this article.

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