RESEARCH ARTICLE

Silencing of Twist Expression by RNA Interference Suppresses Epithelial-mesenchymal Transition, Invasion, and Metastasis of Ovarian Cancer

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Abstract

Purpose: This study aimed to explore the role of the Twist gene in the epithelial-mesenchymal transition of ovarian cancer. Methods: An RNA interference plasmid expressing a small interfering RNA (siRNA)-targeting Twist (Twist siRNA vector) was designed, constructed, and transfected into the human ovarian cancer cell line A2780. Transfection efficiency was assessed under a fluorescence microscope. Changes in the expression of Twist mRNA in A2780 after transfection with the pGenesil Twist shRNA plasmid were analyzed through RT-PCR. MTT assays and adhesion experiments were applied to determine changes in proliferation and adhesion ability of A2870 after transfection with the Twist shRNA plasmid. Changes in the expression of the E-cadherin and N-cadherin proteins in A2780 after transfection with the Twist shRNA plasmid were analyzed using Western blotting. Result: The restructuring plasmid pGenesil-Twist shRNA was constructed successfully. After 48 h of culture, 80% of the cells expressed high-intensity GFP fluorescence and stability. The expression of Twist decreased significantly after the transfection of the Twist shRNA plasmid (P<0.05). Proliferation of the transfected Twist shRNA cells showed no difference with that of the A2780-nontransfection or A2780-si-control groups (P>0.05) but the adhesion ability of A2780 decreased dramatically (P<0.05). Expression of the E-cadherin protein increased, whereas that of the N-cadherin protein decreased compared with that in the A2780-nontransfection or A2780-si-control groups (P<0.05). Conclusion: Twist is essential for epithelial-mesenchymal transition, invasion, and metastasis of ovarian cancer.

Keywords: Twist - RNAi - ovarian cancer - epithelial methenchymal transition

Asian Pacific J Cancer Prev, **13 (9)**, 4435-4439

Introduction

Metastasis is a complex multi-step process. The abdominopelvic cavity is prone to ovarian cancer cell invasion and dissemination. However, the mechanism by which ovarian cancer cells invade and metastasize remains unclear. Epithelial-mesenchymal transition (EMT) plays an important role in early embryogenesis (Thiery, 2002). During EMT, epithelial cells lose their polarity and acquire the locomotive phenotype of mesenchymal cells. In vitro and in vivo studies showed that EMT is important in the infiltration and dissemination of epithelial tumor cells (Radisky, 2005; Puisieux, 2009). EMT activation occurs during the progress, infiltration, and invasion of epithelial tumor cells. This event is essential in the development and metastasis of malignant tumor cells. The expressions of epithelial markers (such as E-cadherin) and mesenchymal markers (such as N-cadherin) are also changed (Yang et al., 2004; Lee et al., 2006). However, no direct evidence of EMT in vivo is currently available. The downregulation of the epithelial marker E-cadherin and the upregulation of the mesenchymal marker N-cadherin indirectly indicate EMT. Previous studies on the embryonic development of Drosophila showed that the Twist gene can regulate cell migration and tissue remodeling, and induce mesoderm development (Soo et al., 2002). The expression of the Twist gene is upregulated in several kinds of neoplasms (Valsesia-Wittmann et al., 2004; Kwok et al., 2005; Hasselblatt et al., 2009; Valdés-Mora et al., 2009). Yoshida et al. (2009) found that the expression of the Twist protein is higher in ovarian cancer tissues than in benign and borderline ovarian tumors. A recent study has confirmed that the Twist gene is closely related to EMT and neoplasm metastasis (Niu et al., 2007). Moreover, the Twist gene is believed to be the key regulatory factor of EMT. At present, intensive studies on the relationship between the Twist gene and the EMT, invasion, and metastasis of ovarian cancer are lacking.

Our previous research showed high Twist and N-cadherin expressions but low E-cadherin expression. The high expression rates of Twist and N-cadherin and the low expression rate of E-cadherin were found to be related to

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the grade, stage, and lymph node metastasis of ovarian cancer. This result indicated that the oncogene Twist may be involved in the invasion and metastasis of ovarian cancer and that these processes may be related to EMT. This study aims to discuss the possible role of Twist in promoting the invasion and metastasis of ovarian cancer cells. We applied the RNA interference technique to inhibit the expression of Twist in the ovarian cancer cell line A2780. Changes in the proliferation, invasion, and adhesion properties of A2780 in vitro as well as changes in the expressions of E-cadherin and N-cadherin were determined. The results of the present study elucidate the possible role of Twist in the EMT, invasion, and metastasis of ovarian cancer cells.

Materials and Methods

Vector construction

According to the sequence of Twist mRNA genes on GenBank (GenBank Accession Number: NM_000474), two target set sequences with a length of 21 bp and a short hairpin structure were designed following the principles of Tuschl: the 777th interference sequence (shRNA1): GCTGAGCAAGATTCAGACCCT; the 845th interference sequence (shRNA2): GCGACGAGCTGGACTCCAAGA; and the si-control vector (control siRNA vector expressing siRNA that does not match any known human coding mRNA) sequence: GACTTCATAAGGCGCATGC. The plasmid was extracted after annealing, connecting, and transforming the competent cell DH5α. The recombinant vectors were then identified through enzyme digestion and gene sequencing. The target recombinant plasmids were obtained when the results are the same with the designed result of appraisal and sequencing. The obtained products were named pGenesil Twist shRNA1 plasmid and pGenesil Twist shRNA2 plasmid.

Transfection

A2780 cells were transfected with pGenesil Twist shRNA1 plasmid, pGenesil Twist shRNA2 plasmid, and pGenesil si-control plasmid. After cultivation for 24, 48, and 72 h, the cells were collected. Transfection was observed under a fluorescence microscope. RT-PCR was used to detect the expression of the Twist mRNA in the transfected cells.

MTT assay

The test was divided into three groups (A2780-nontransfection, A2780-si-control, and A2780-Twist-shRNA) with six holes each. The cells were cultured for 24, 48, and 72 h. A microplate reader at a wavelength of 750 nm was used to measure the optical density value of each hole.

Transwell chambers

Peridium and hydrating basement membrane, the number of vaccination cells was 1×10^5; the division of groups was as that of 1.4.1, 6 wells each group, and cultured for 48 h. Ten high power fields of each sample were counted randomly.

Adhesion measurement

Non-adhesive cells were washed with D-hanks solution. The MTT colorimetric method was used to measure the light absorption value (A value) of each porocyte. The A value of the matched basilar membrane anchorage-dependent cell of the bovine serum albumin (BSA) group was obtained. The adhesion rate of the three cell lines in the Matrigel group was calculated as follows: Adhesion rate (%) = [(A value of the cells in the experimental group/A value of the cells in the BSA group)-1] × 100

Western blot

Western blot analysis results revealed that the expressions of Twist and N-cadherin in ovarian cancer tissues were higher than in normal ovarian tissues. By contrast, the expression of E-cadherin was reduced in ovarian cancer tissues. Relative gray value showed that the P-values were all less than 0.05.

Results

Vector construction

Both enzyme digestion and gene sequencing showed that the designed shRNA segment was successfully connected to the pGenesil vector.

Transfection rate

Green fluorescence (GFP) was observed through the microscope in the successfully transfected cells. The transfection rates of the A2780 cells after 24 and 48 h of culture were 70% (Figure 1) and 80%, respectively.

RT-PCR

Through the analysis of the gray value of each group, no significant differences were observed between the A2780-nontransfection group and the A2780-si-control group. Significant differences were noted among the A2780-Twist-shRNA1 group, the A2780-Twist-shRNA2 group, and the A2780-nontransfection or A2780-si-control

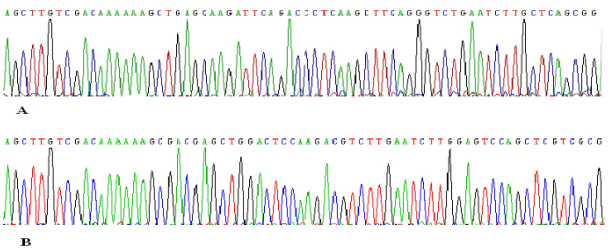

Figure 1. The Observation Under the Simple Microscope (A) and the Observation Under the Fluorescence Microscope (B)

Table 1. The Expression of the Twist mRNA in cell A2780 after Transfected pGenesil-Twist shRNA Interference Plasmid

Cell	Twist	GAPDH	Twist/GAPDH
A2780-nontransfection	105.4±10.5	99.8±8.9	1.04±0.028
A2780-si-control	114.5±10.2	108.5±12.6	1.10±0.026
A2780-Twist-shRNA1	40.5±8.1	100.9±11.8	0.38±0.033
A2780-Twist-shRNA2	55.6±7.9	104.8±13.1	0.51±0.029

Table 2. The Result of the MTT Test about the A2780 Cell after Transfected the Twist shRNA Plasmid (n=6, $\bar{\chi}$±S), the Comparisons among the Three Groups, P >0.05

Cell	24 h	48 h	72 h
A2780-nontransfection	0.481±0.021	0.615±0.064	0.889±0.068
A2780-si-control	0.447±0.028	0.705±0.051	0.856±0.035
A2780-Twist-shRNA	0.475±0.025	0.605±0.041	0.712±0.034

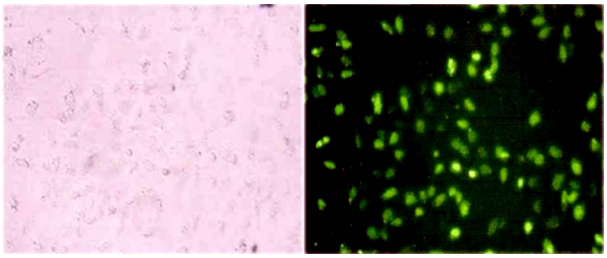

Figure 2. The Comparison of the Adhesion Rate of the Three Groups, *P<0.05

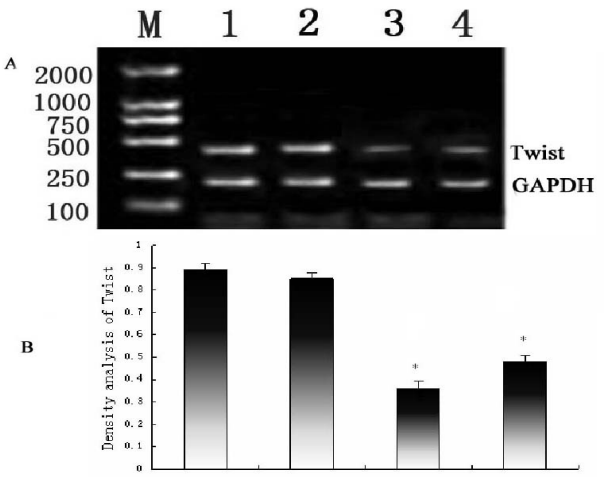

Figure 3. A) The Expression of E-cadherin of Cell A2780 after Transfected Twist shRNA by Western-Blot. 1: A2780-nontransfection group 2: A2780-si-control group 3: A2780-Twist-shRNA. **B) The Expression of N-cadherin of Cell A2780 after Transfected Twist shRNA by Western-Blot.** 1: A2780-nontransfection group; 2: A2780-si-control group; 3: A2780-Twist-shRNA

group (P<0.05; Table 1). The interference efficiencies of shRNA1 and shRNA2 against Twist mRNA were 65% and 56%, respectively.

MTT assay

Based on the MTT test results, the proliferation of the transfected Twist shRNA cells did not show any significant difference with that of the A2780-nontransfection or A2780-si-control group (P>0.05; Table 2).

Adhesion ability

Compared with A2780-nontransfection and A2780-si-control groups, the adhesion ability of the A2780-Twist-shRNA group decreased significantly (P<0.05; Figure 2).

Western blot analysis

The expression of E-cadherin increased significantly in the A2780-Twist-shRNA group than in the A2780-nontransfection and A2780-si-control groups (P<0.05).

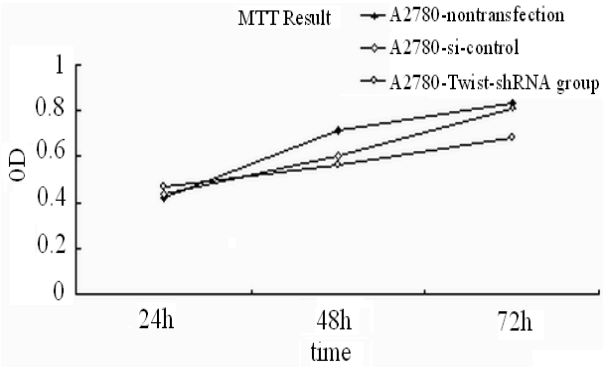

Figure 4. The Proliferation Condition or the Three Groups with MTT Method. a. A2780-nontransfection; b. A2780-si-control; c. A2780-Twist-shRNA group

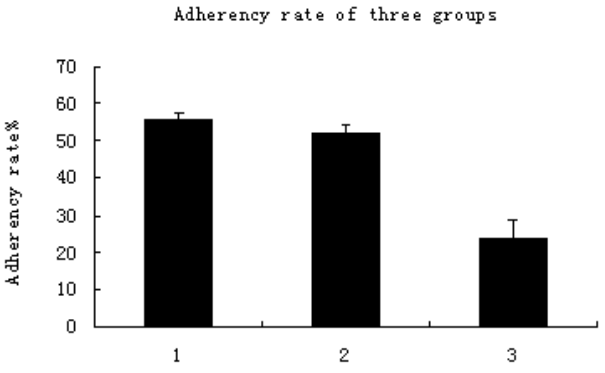

Figure 5. The Comparison of the Adhesion Rate of the Three Groups, *P<0.05

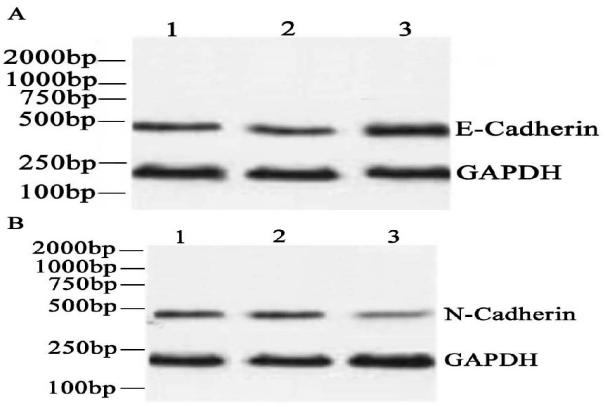

Figure 6. A) The Expression of E-cadherin of Cell A2780 after Transfected Twist shRNA by Western-Blot. 1: A2780-nontransfection group 2: A2780-si-control group 3: A2780-Twist-shRNA. B) The expression of N-cadherin of cell A2780 after transfected Twist shRNA by Western-blot.1: A2780-nontransfection group; 2: A2780-si-control group; 3: A2780-Twist-shRNA

By contrast, the expression of N-cadherin decreased significantly. No significant differences in the expressions of E-cadherin and N-cadherin were found between the A2780-nontransfection and A2780-si-control groups (P>0.05; Figure 6).

Discussion

Ovarian cancer is one of the three malignant tumors of the female reproductive system, the mortality of which ranks the first in the gynecological malignancy. In recent years, the incidence of ovarian cancer has increased. The

abdominopelvic cavity is prone to ovarian cancer cell invasion and metastasis. Thus, inhibition of the invasion, spread, and transfer of ovarian cancer cells has been the focus of different research groups for clinical treatment.

During EMT, epithelial cells are converted into interstitial cells under specific physiological and pathological conditions. Through EMT, epithelial cells lose their polarity and achieve the locomotive phenotype of interstitial cells (Hay, 2004). EMT is important in embryonic development and histogenesis (Boyer et al., 2000). Recent studies have shown that EMT is essential in the development, infiltration, and invasion of epithelial tumors. The expressions of epithelial markers (such as E-cadherin) are transformed into the expressions of mesenchymal markers (such as N-cadherin) (Yang et al., 2004; Lee et al., 2006).

Twist is the key transcriptional regulator in the embryonic development of tissue reconstruction. Twist, an oncogene overexpressed in several epithelial cancers, was found to be closely correlated with EMT and tumor metastasis (Desprez et al., 2003; Kang and Massague, 2004; Yang et al., 2004). Twist is necessary in the transfer of adenocarcinoma cells from the breast to lungs (Yang et al., 2004). Noriyuk studied the liver cancer (Matsuo et al., 2009) and found no obvious expression of Twist on the slice edge without tumor. Twist promotes the transmission of liver cells through EMT, demonstrating the high motion ability of Twist. Moreover, the strong positive expression of Twist is related to the recurrence of laryngeal squamous carcinoma, whereas the tumor of negative Twist is relatively smaller and often detected in an earlier stage, with a relatively high five-year survival rate (Jouppila-Mättö et al., 2011). The expression of Twist in rectal carcinoma tissue is obviously higher than that in non-tumor colonic mucosa. Its expression in patients with lymph node metastasis is also high (Yoshida et al., 2009). These findings suggest that Twist plays a role by adjusting EMT and that it has a close relationship with the development and metastasis of malignancy.

Previous studies reported that a relationship exists between Twist and ovarian cancer. However, intensive studies remain scarce. Yoshida et al. (2009) revealed that the expression of Twist is higher in epithelial ovarian tumors than in normal ovarian surface epithelial cells. The expression of the Twist protein sequentially increases in benign, borderline, and malignant ovarian tumors. Its expression is also increased dramatically in poorly differentiated ovarian tumors. Furthermore, Twist is related to the FIGO stage of ovarian tumor. Particularly, it is related to the occurrence and spread of ovarian tumors to the peritoneum. RNA interference may be used to inhibit effectively the expression of target gene mRNA (Haney, 2007; Yu, 2007). RNAi technology was applied using eukaryotic expression vector Genesil2 to construct the pGenesil2-Twist shRNA vector that targets and inhibits Twist gene expression in the ovarian cancer cell line A2780. Twist mRNA expression was then analyzed. The result showed that the transfected Twist shRNA can obviously inhibit the expression of Twist mRNA. Consequently, Twist silencing affected the proliferation ability, adhesion ability, and E-cadherin and N-cadherin expressions of the ovarian cancer line in vitro. The results showed that the inhibition of the Twist gene did not affect the proliferation capacity of the ovarian cancer cell line A2780 but decreased its adhesion to the matrix. After A2780 was transfected with the Twist-shRNA plasmid, the expressions of E-cadherin and N-cadherin increased and decreased, respectively. The results of the present study agree with the findings of previous studies on liver cancer and laryngocarcinoma (Matsuo et al., 2009). However, no conclusion was derived in terms of the effect of Twist on the proliferation ability of the tumor cells. Some researchers found that Twist can promote tumor cell proliferation (Hasselblatt et al., 2009), whereas others found that it can promote the invasion of tumor cells and has no effect on cell multiplication (Matsuo et al., 2009). Hasselblatt et al. (2009) found that Twist is expressed in choroidal carcinoma and can promote the proliferation and invasion of choroidal epithelial cells. Noriyuki found that overexpressed Twist cells could enhance locomotivity. However, this locomotivity was decreased significantly after Twist-shRNA treatment. Thus, Twist promoted the transfer of liver cells through EMT (Matsuo et al., 2009). Therefore, we can determine that Twist gene silencing can inhibit the adhesion ability of the ovarian cancer cell line A2780. The expression of the epithelial marker E-cadherin increased, whereas that of the mesenchymal marker N-cadherin decreased. Twist gene inhibition can suppress the EMT, invasion, and metastatis of ovarian cancer cells.

Our research showed that the inhibition of the Twist gene can decrease EMT and the adhesion ability of the ovarian cancer cell line A2780 in vitro. However, it did not affect the proliferation capacity of the cell line. Thus, Twist is related to the metastasis and EMT of ovarian cancer cells in vitro but not to their proliferation ability.

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