Original Article
TROP-1/Ep-CAM and CD24 are potential candidates for ovarian cancer therapy

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Abstract: To clarify the possible roles of epithelial cell adhesion molecule (TROP-1/Ep-CAM) and CD24 molecule (CD24) in ovarian tumorigenesis, and explore the possible mechanism underlying this disease. Recombinant eu-karyotic expression vectors pCIneo-TROP-1/Ep-CAM and pCIneo-CD24 were transfected into human normal ovarian surface epithelia cell line IOSE-80 respectively, with IOSE-80 cells transfected with the empty vector pCIneo as control. MRNA and protein expression of TROP-1/Ep-CAM and CD24 were detected by RT-PCR and Western blotting, respectively. Cell migration was assayed by trans-well inserts; cell proliferation and adhesion were analyzed by CCK-8 Cell Counting kit; cell cycle and cell apoptosis analysis were performed by flow cytometer. The expressions of TROP-1/Ep-CAM and CD24 were obviously up-regulated in TROP-1/Ep-CAM group and CD24 group compared to that in control group (P < 0.01). Cells of TROP-1/Ep-CAM group and CD24 group was significantly promoted migratory and proliferation abilities, but inhibited cell apoptosis and adhesive than that of control group (P < 0.05). Besides, the number of the cells in G1 and G2 stages was significantly lower in two disease groups than that in control group (P < 0.05). TROP-1/Ep-CAM and CD24 may play key roles in the progression of ovarian cancer through promoting migration, proliferation, inhibiting cell apoptosis and adhesion, and disturbing cell cycle. They may be used as specific therapeutic targets in the treatment of ovarian cancer. However, further experiments are still needed to confirm our results.

Keywords: Ovarian cancer, TROP-1/Ep-CAM, CD24, apoptosis, cell migration

Introduction

Ovarian cancer is one of the most common female malignancies and is easy to metastasis to some common areas such as the lymph nodes, lining of the abdomen, lung, and liver [1]. The risk factors include inherited genetic risk, fertility medication, hormone therapy after menopause and obesity [2]. In the United States, the overall five year survival rate is 45%, and outcomes are worse in the developed countries [3]. The morbidity and mortality of ovarian cancer are high worldwide due to easy metastasis and hard diagnosis [4, 5]. Therefore, there is a pressing need to elucidate the underlying mechanism of ovarian cancer for developing potent therapies.

Previous studies have demonstrated that traditional treatment methods on ovarian cancer are mainly involved surgery, chemotherapy and sometimes radiotherapy [6]. However, disadvantages of these treatments are the side effect on patients such as proteinuria, high blood pressure and bowel perforation [7]. Recently, the roles of therapeutic targets have been highlighted in the physiological mechanism and therapeutic intervention of ovarian cancer, such as heparin-binding EGF-like growth factor [8], EphA2 [9], and E-cadherin [10]. Although tremendous efforts have been made to discover novel targets for ovarian cancer treatment, the present knowledge seems to be insu.

Recently, some key genes have been reported to play important roles in ovarian cancer [11, 12]. CD24 molecule (CD24) is a glycosylated glycosyl phosphatidyl inositol (GPI)-linked cell surface protein, which is expressed on mature granulocytes and in many B cells [13, 14]. Epithelial cell adhesion molecule (TROP-1/Ep-CAM) encodes an epithelial transmembrane
glycoprotein over-expressed in various carcinomas [15]. TROP-1/Ep-CAM and CD24 were the DEGs between ovarian carcinomas and normal ovarian epithelium, they were up-regulated in ovarian cancer, indicating its involvement in the pathogenesis of ovarian cancer [16, 17]. Although former study reveals that TROP-1/Ep-CAM and CD24 may be potential therapeutic target for ovarian cancer [18]. However, the exact role of TROP-1/Ep-CAM and CD24 over-expression and the functional importance in development of ovarian cancer still remains unclear [18].

In the present study, we analyzed the expression of TROP-1/Ep-CAM and CD24 in human ovarian surface epithelium cell line IOSE-80 and investigated the impact of TROP-1/Ep-CAM and CD24 on cell migration, adhesion, proliferation, apoptosis and cell cycle. This study aimed to investigate the potential roles of TROP-1 and CD24 in ovarian cancer development and progression. Overall, our results would provide novel insights into the pathogenesis and treatment of ovarian cancer.

**Materials and methods**

**Cell culture**

The human ovarian surface epithelial cell line of IOSE-80 (BIOLEAF, Shanghai, China) was cultured in medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and antibiotics (100 Ag/mL streptomycin and 100 units/mL penicillin). The ovarian cancer cell line OVCAR-5 was cultured in McCoy's 5A medium containing 10% FBS and antibiotics. All cultures were incubated at 37°C with 5% CO₂: 95% air.

**Expression constructs and cell transfection**

Full-length cDNAs for Ep-CAM and CD24 were PCR amplified from ovarian cancer cell line OVCAR-5 after RNA extraction and reverse transcription. For amplification of Ep-CAM, the primers used were sense: 5'-AGAGAATTCCATGGTGATCCCGTGCGATG-3' and anti-sense: 5'-GAGGTCGACTTACCTACCTACCTGGCATG-3'. For CD24 amplification, the primers used were sense: 5'-AGAGAATTCCATGGTGATCCCGTGCGATG-3' and anti-sense: 5'-GAGGTCGACTTACCTACCTACCTGGCATG-3'. The obtained PCR fragments of Ep-CAM and CD24 were cloned into pCIneo (Promega, Madison, WI) with Sal I and EcoR I, and all the constructs were sequenced. Transfections were done using Fugene according to the manufacturer's instructions (Roche, Indianapolis, IN). IOSE-80 cells were transfected with pCIneo-EpCAM, pCIneo-CD24 and pCIneo-EV (empty vector) plasmids. IOSE-80 clones were screened and maintained in medium supplemented with G418 (500 Ag/mL).

**Quantitative real-time PCR**

To validate the transfection efficiency, quantitative RT-PCR was performed with an ABI Prism7000 Sequence Analyze (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. Primers for TROP-1 were obtained from Applied Biosystems as assay on demand products. Assay ID was Hs00158980_m1 (TROP-1). CD24 primer sequences were as follows: sense: 5'-cccaacgtgttactgttaattcctcaa-3'; anti-sense: 5'-gaacagcaatagctcaacaatgtaaac-3'. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: sense primer: 5'-CATGAGAAGTATGCAACAGGCTC-3'; anti-sense: 5'-AGTCCTCCACGATACCAAATG-3'. GAPDH was used as an internal control.

**Western blotting assay**

Cells cultures were washed twice with ice-cold PBS and cells were lysated using lysis buffer: 10% glycerol, 62.5 mmol/L Tris-HCl (pH 6.8) and 2% SDS. Then the samples were diluted with 2 × SDS-PAGE loading buffer (1:1), followed by thermal denaturation at 100°C for 5 min. After cooling down, the supernatants were collected by centrifugation at 12,000 rpm at 4°C for 10 min. The protein concentration was quantitated by a biinchoninic acid (BCA) assay (Pierce, Sunshine Biotechnology, Nanjing, China). The protein samples were separated by a 10% SDS-polyacrylamide gel (Invitrogen) and transferred to nitrocellulose membranes. After blocking for 2 h, the membranes were incubated overnight at 4°C with primary antibodies at the following dilutions: anti-TROP-1, 1:200; anti-CD24, 1:250 (Zymed, San Francisco, CA). After washing with TBST for 3 times, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) (anti-mouse or anti-rabbit IgG: 1:10,000; Amersham Pharmacia Biotech, Piscataway, NJ). Reactive bands were detected by enhanced chemiluminescence reagent (Pierce, Rockford,
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IL, USA). GAPDH was used as the internal control.

**Cell migration assays**

Cell migration was assayed by trans-well inserts coated with 8 µm pore membrane (BD Biosciences, San Jose, CA, USA) as described previously [19]. The transfected cells were allowed to grow to about 80% sub-confluence and then serum-starved overnight. After detachment with trypsin, the cells were washed with PBS and suspended in serum-free medium. Subsequently, an aliquot of 100 µL of cell suspension (3 × 10^5 cells/ml) was added to the upper chamber. The membranes were coated with 0.01% collagen type-I in 0.01% N HCL (Sigma). The lower chamber was filled with 700 µL of RPMI-1640 medium with 15% FBS. After 48 h incubation in a 5% CO_2 incubator at 37°C for 48 h, the membranes were peeled off. The cells remained on the upper side of the membranes were wiped off using cotton swabs, whiles the other cells migrated to the lower side were fixed with 500 µL methanol for 10 min at -20°C and then stained with 200 µL 0.1% crystal violet for 30 min at 37°C. Finally, images of five different fields (× 100) were captured from each membrane and the number of migrated cells was counted for each field. The numbers of migrated cells of five fields were averaged for each experimental condition.

**Cell adhesion and proliferation assay**

Cell adhesion assay was performed as described previously [20]. In brief, 96-well flat-bottom culture plates were coated with 60 µL of Matrigel diluted in serum-free DMEM medium (1:5) with 5% CO_2 at 37°C for 4 h in advance. The cells were harvested with 1 mL trypsin-EDTA solution 48 h after infection, then washed twice and resuspended in DMEM medium. Then, the cells were added to each well (5 × 10^4/well) in quintuplicate and incubated at 37°C for 3 h. Following that, the 96-well plates were washed twice with PBS to remove unbound cells followed by addition of a 100 µL aliquot of fresh medium. The remaining adhesive cells on the plate were counted by a CCK-8 Cell Counting kit (Dojindo Laboratories, Kumamoto, Japan). Finally, these 96-well plates were examined at 450 nm using a format plate reader 2.5 h later. The results were calculated as follows: Adhesion rate = (mean OD of treated cells)/mean OD of corresponding control) × 100%.

Cell proliferation assay was also conducted with CCK-8 Cell Counting kit [21]. Cells in the DMEM medium were seeded in 96-well plates at approximately 5 × 10^3 cells/well. At the indicated time points (0 h, 24 h, 48 h, 72 h and 96 h), each well were added with 10 µL of CCK-8 solution and then incubated for 2.5 h, followed by measuring absorbance at 450 nm. These experiments were done in triplicates and repeated at least twice.

**Cell apoptosis assays**

Cells after LV infection were incubated at a density of 5 × 10^5/ml for 48 h and then harvested with EDTA free-trypsin for apoptosis analysis using AnnexinV-PE/7-AAD apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the instructions of the manufacturer [22]. Briefly, after washing twice with cold PBS and centrifugation at 2000 rpm for 5 min, the cells were resuspended in 50 µL of binding buffer and then incubated with 5 µL of 7-AAD in dark for 15 min at room temperature. Subsequently, the cells were incubated with 450 µL of binding buffer and 1 µL of Annexin V-PE for another 15 min in dark followed by immediate detection with FACS Calibur flow cytometer (Beckton-Dickinson, San Jose, CA, USA).

**Cell cycle analysis**

Prior to harvesting, the cells after LV infection were incubated at a density of 5 × 10^5/ml for 48 h. Then, the cells (1-5 × 10^6/ml) were washed twice with ice-cold PBS, resuspended in 500 µL of PBS and fixed with 1.5 mL of pre-cooled 100% ethanol overnight at 4°C sequentially. After washing twice with PBS, the fixed cells were centrifuged (1000 rpm × 5 min) to remove the ethanol. After concentration adjusting to 1-10 × 10^6 cells/mL, the cells were incubated with 150 µL of RNaseA (250-500 µg/mL) for 30 min at 37°C followed by the incubation with an addition of 100 µL of propidium iodide (PI, Sigma, CA, USA) for 30 min in dark at 4°C. Their DNA content was analyzed by using a FACS Calibur flow cytometer (Beckton-Dickinson, San Jose, CA, USA) with an excitation wavelength of 488 nm [23].

**Statistical analysis**

Data were shown as mean ± SD, and all statistical analyses were conducted using SPSS 13.0 (SPSS Inc, Chicago, IL, USA).
Differences among the experimental groups were analyzed using Student’s t-test or one-way ANOVA with LSD post hoc test. Statistical significance was set at $P < 0.05$. 

Figure 1. TROP-1 and CD24 expression in IOSE-80 cells. Western blotting results of TROP-1 and CD24 expression in IOSE-80 cells. GADPH was regarded as the internal reference. IOSE-TROP-1, cells stably transfected with TROP-1. IOSE-CD24 is stable transfected with CD24. Empty vector control cells (IOSE-EV) do not express any detectable TROP-1 and CD24 was used as loading control.

Figure 2. Effect of TROP-1 and CD24 on migration of IOSE-80 cells. A-C: Photographs of migrated IOSE-80 cells in control, CD-24 and TROP-1 groups (100×). D: The statistical results of migrated IOSE-80 cells in control, CD-24 and TROP-1 groups. The migrated IOSE-80 cells in CD-24 and TROP-1 groups were increased compared to that in control group (**$P < 0.01$, compared to control group).
Figure 3. Effect of TROP-1 and CD24 on adhesion and proliferation of NHEKs. A: Cell adhesion analysis of control, CD-24 and TROP-1 groups. B: Cell proliferation analysis of control, CD-24 and TROP-1 groups.

Figure 4. Impact of TROP-1 and CD24 on cell apoptosis of IOSE-80 cells. A: Cell apoptosis analysis of control, CD-24 and TROP-1 groups with FACS Calibur flow cytometer. B: The statistical results of the proportion of apoptotic and viable cells in control, CD-24 and TROP-1 groups (**P < 0.01, compared to control group).
Figure 5. Effect of TROP-1 and CD24 on cell cycle of IOSE-80 cells. A-C: Cell cycle analysis of IOSE-80 cells in control, CD-24 and TROP-1 groups with FACS Calibur flow cytometer. D, E: The statistical results of the proportion of cells in S, G1 and G2 stages in three groups (*P < 0.05, **P < 0.01, compared to control group).
Results

**TROP-1 and CD24 expression in IOSE-80 cells**

The PCR results showed that IOSE-TROP-1 expressed high levels mRNA of TROP-1 and clones IOSE-CD24 expressed high levels mRNA of CD24. The control cells (IOSE-EV) transfected with the empty vector did not express these mRNAs (Figure 1A). Similarly, selected stable clones using TROP-1 and CD24-specific antibodies showed that clone IOSE-TROP-1 expressed high levels of TROP-1 and clones IOSE-CD24 expressed high levels of CD24. The control cells (IOSE-EV) transfected with the empty vector did not express these proteins (Figure 1B, 1C).

**Cell migration, adhesion and proliferation assay**

Trans-well analysis showed that both migrate IOSE cells transfected with TROP-1 and CD24 were statistically high compared with the control cells ($P < 0.01$) (Figure 2). As shown in Figure 3A, the cell adhesion in TROP-1 and CD24 groups were significantly inhibited compared with that in control group ($P < 0.05$). Moreover, as shown in Figure 3B, TROP-1 and CD24 also promoted cell proliferation.

**Cell apoptosis assay**

Figure 4A-C depicted that the proportion of early apoptosis cells in total cells was significantly decreased in the TROP-1 and CD24 groups compared with that in control group ($68.01\%$ vs. $0.76\%$ and $0.92\%, P < 0.01$). Therefore, TROP-1 and CD24 appear to inhibit the apoptosis of NHEKs.

**Cell cycle assay**

As shown in Figure 5A-E, the cell cycle in TROP-1 and CD24 groups were distinctive from that of control group. Cells in S stage were markedly increased ($25.90\%$ and $26.07\%$ vs. $18.19\%, P < 0.05$) but the cells in G1 and G2 stage were sharply reduced in TROP-1 and CD24 groups compared with that in control group ($65.29\%$ vs. $71.82\%$ and $71.82\%$ compared with control group, $P < 0.01$). It revealed that TROP-1 and CD24 could inhibit the increase of cells in G1 and G2 stages.

Discussion

Ovarian cancer is one of the most common female malignancies, which is easy to metastasis and with high morbidity and mortality [1]. Previous paper have predicted that key molecular of TROP-1/Ep-CAM and CD24 may play crucial roles in ovarian cancer therapy [ENREF_7]. The current study analyzed the impacts of TROP-1/Ep-CAM and CD24 on cell function in normal human ovarian surface epithelial cell. Our data showed that overexpression of TROP-1/Ep-CAM and CD24 could promote ovarian cancer cell migration and proliferation, inhibit cell apoptosis and adhesion, and disturb cell cycle.

Our data showed that overexpression of TROP-1/Ep-CAM and CD24 could promote migration of IOSE-80 cells. Study referred that ovarian cancer was related to genes involving promoting cell migration and invasion [25]. Besides, the over-expression of TROP-1/Ep-CAM and CD24 also affected the cell cycle of IOSE-80 cells, promoted cell proliferation and inhibited cell apoptosis and adhesion. It indicated that the over-expression of TROP-1/Ep-CAM and CD24 might promote the progress of ovarian cancer by promoting migration and proliferation, and inhibiting apoptosis and adhesion.

CD24 over-expression has been reported at the RNA level in ovarian cancer [24] and overexpression of CD24 has been reported to be a prognostic marker for ovarian cancer. CD24 is an adhesion receptor on activated endothelial cells, its expression may contribute to the metastatic capacities of ovarian tumor cells [26, 27]. Studies showed that CD24 initiates cell proliferation and survival [28, 29]. Moreover, some studies showed that CD24 overexpression is linked to increased invasion and lymph node metastasis in breast cancer [30, 31]. Furthermore, studies also showed that CD24 was a candidate target for the therapy of cancers [32, 33]. Based on our results, we speculated that the significant effect of overexpression of CD24 was associated with ovarian cancer progression and metastasis through affecting cell migration, adhesion, proliferation and apoptosis.

Ep-CAM has been reported to be closely associated with malignant proliferation in most adult epithelial tissues [34], as well as related to cell adhesion and tumor metastasis [35]. Besides, Ep-CAM has attracted some attention as a target for immunotherapy in carcinomas [36]. Previous paper showed that Trop-1/
Ep-CAM was overexpressed in many cancers such as breast cancer [37], ovarian cancer [38], and lung cancer [39]. Also, Ep-CAM is a prognostic marker for ovarian cancer [40]. Litvinov showed that Ep-CAM in cervical squamous epithelia correlated with an increased proliferation [41]. Our data displayed that overexpression of TROP-1/Ep-CAM could inhibit ovarian cancer cell apoptosis and adhesion, disturbs cell cycle and promotes cell migration and proliferation, indicating that TROP-1/Ep-CAM may play crucial roles in ovarian cancer progression and metastasis by affecting cell migration, adhesion, proliferation and apoptosis in ovarian cancer.

In conclusion, our study suggests that overexpression of TROP-1/Ep-CAM and CD24 could contribute ovarian cancer metastasis and progression. The two key proteins may disturb the cell cycle of IOSE-80 cells, promote cell proliferation and migration, and inhibit cell apoptosis and adhesion. TROP-1/Ep-CAM and CD24 may be a novel and potentially effective therapeutic target for ovarian cancer patients. However, further experimental studies are still needed to investigate the comprehensive mechanism of the two key molecules in ovarian cancer.

Disclosure of conflict of interest

None.

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