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Effect of WFDC 2 silencing on the proliferation, motility and invasion of human serous ovarian cancer cells *in vitro*

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ABSTRACT

Objective: To investigate effect and possible mechanisms of silencing human WFDC2 (HE4) gene on biological behavior changes as cell proliferation, apoptosis, movement and invasion of human serous ovarian cancer cell line SKOV3. **Methods:** Lentiviral WFDC2 gene sequence of small interfering siRNA was stably transfected into SKOV3 identified by Q−PCR and western−blot. Obtained SKOV3 stable strains with silenced HE4 were measured by proliferation, apoptosis, migration, and invasion. **Results:** Gene sequencing showed that the oligonucleotides were successfully inserted into the expected site. After silencing HE4 in the SKOV3, proliferation was significantly inhibited ($P<0.05$). G_0/G_1 phase was arrested by the cell cycle ($P<0.01$) and capacity of the migration and invasion decreased significantly ($P<0.01$). Slight early apoptosis ratio and no change of late apoptosis were found without change of Caspase−3 or Bcl−2 protein. Proteins involed in ERK pathway as phosphorylated protein as p−EGFR, p− ERK decreased and protease protein involved in tissue remoding as matrix metalloproteinases MMP−9, MMP−2 and cathepsin B decreased compared with control group. **Conclusions:** HE4 gene plays an important role in regulating proliferation, apoptosis, migration, invasion of serous ovarian cancer cells by ERK pathway and protease system. Its role in apoptosis needs to be further explored, and it may be a potential target for serous ovarian cancer.

1. Introduction

Ovarian cancer is a women tumor with high malignant degree, its five−year survival rate was less than 30%. The poor survival rate of ovarian cancer was mainly due to the lack of effective screening methods and the early patients were without obvious symptoms. Once diagnosed, patients were already in advanced stage[1]. The CA125 was lack of

sensitivity and specificity, which is not a good indicator. It is found in recent years that human epididymis−specific protein 4 (HE4, encoded by WFDC2 gene) will be a promising new markers of ovarian cancer[2], which is important in the diagnosis[3], monitoring[4] and prognosis[5] of the ovarian cancer. This new diagnostic indicators combined with CA125 constitute the ROMA index, which helps to assess the risk of ovarian epithelial cell carcinoma of women with a pelvic mass pre−menopausal/post−menopausal. Studies have shown that the HE4 were with high expression in ovarian serous adenocarcinoma, and with weak expression in benign ovarian tumors and endometriosis[6]. Hereby, we speculated that HE4 gene might plays an important role in the occurring and developing of the ovarian cancer. So

clarifying WFDC2 gene function may help us to identify a new therapeutic target in ovarian cancer. Unfortunately, the function of WDC2 gene in ovarian cancer is unclear then.

Some short fragment of double—stranded RNA can promote specific gene mRNA degradation to specifically blocking gene expression *in vivo*, and induce cells exhibit specific gene deletion phenotype, which was called RNA interference (RNAi)[7]. The landmark discovery of RNAi can specifically inhibit the expression of the target gene. In order to explore the function of WFDC2 gene in human serous ovarian carcinoma, we apply lentiviral small interfering specific to inhibit the WFDC2 gene expression in human serous ovarian carcinoma cell line SKOV3, and observed the change of biological characteristics as cell proliferation, apoptosis, cell cycle and ability of invasion and migration.

2. Materials and methods

2.1. Materials

Human serous ovarian cancer cell line SKOV3 was provide by Cells Center of the Peking University Health Science Center; siRNA fragments and control fragment and the lentiviral vector pLKO.1 were provided by the Beijing Qianjing Kechuang Biotechnology Co., Ltd.; HE4 mouse anti—human antibodies were purchased from Abcam; Human HE4 primers were purchased from Invitrogen; Restriction endonuclease *Age* Ⅰ (ACCGGT) and *Eco*R, the small amount plasmid extraction kit Gel Extraction kit were purchased from TianGen; The packaging virus were purchased from the Beijing prospects Kechuang. *Age* Ⅰ (NEB, #R0552S), *EcoR* Ⅰ (NEB, #R0101S), buffer 2 (NEB, #B7002S), solution Ⅰ (Takara, D6022), agarose gel purified extraction kit (QIAGEN, # 28704), 1.5 mL microfuge tube (Biologix, Item No: 80—1500).

2.2. siRNA design, primer design and synthesis

According to the nucleotide sequence (NM_006103.3) of the GenBank human HE4 ,siRNA sequence (5′−3′) 004: 8 strips were designed for the target, the 5th and 8th were proved effective, the above siRNA sequences were synthesized by Invitrogen.

2.3. Serous ovarian cancer cell line SKOV3 culture, passage and cryopreservation

Cell lines were cultivated in the condition of 10% FBS 1640 medium, at 37 ℃, 5% CO_2. The medium was changed every 2 d, when 85% cells were fused, replated at 1: 3 after 0.25% trypsinization was done. Cells were suspended in medium containing 10% DMSO, gradient cryopreservation. HEK293T cells were incubator in DMEM high glucose medium containing 10% FBS at 37 ℃, 5% CO_2 .

2.4. Preparation of lentivirus

293T cell were seeded in 60 mm dishes with the density of 7×10^5/60 mm, and seeded on 60 mm plate with the density of 7×10^5/60 mm, transfected plasmids after 24 h. The plasmid pMD2G and psPAX2 (purchased from addgene company) 1 μg, containing knockdown sequence plko plasmid 2 μg. Transfection reagent 293 Fect 8 μL. Change 5 mL fresh medium after 12—15 h, collected supernatants after 24 h, thus obtained the virus solution.

2.5. siRNA fragments transfection

Passage 2 and 3 cells were taken in this experiment, they were seeded in 24—well plates with 2×10^4/well, and were replaced with 60, 50, 120 nmol /L terminal concentration transfected interference or control siRNA after 24 h; Then cultivated into complete medium after 8 h, and the cells were collected after 48 h of incubation.

2.6. Lentiviral infection and stable cell line obtaining

24 h before infection, the SKOV3 cells were inoculated into 60 mm dishes with inoculum density of 5×10^5 cells/ plate. Next day, lentivirus supernatant that carrying different interference sequence was added, and simultaneously added Polybrene with final concentration of 8 μg/mL. 6 h after infection, removal viral supernatant and exchange for fresh medium, then transferred to 10 cm plate after cultured for 24 h. After cell attachment, replace to fresh medium puromycin with terminal concentration of 1 μg/mL. Observed after two days, control cells not infected with the virus have all died. Puromycin resistant cells were contain HE4 interference sequence now. Collected some cells, extracted the proteins, and use immune blotting to analysis HE4 knock down cell lines.

2.7. Western—blot identification

1 × SDS sample loading buffer [62 mmol/L Tris—HCl (pH6. 8), 2% SDS, 10% glycerol, 50 mmol/L of DTT, 1% bromophenol blue] were used to collect cells of the interference group or control group respectively, 4 ℃ ultrasonication, boiled at 100 ℃ for 5 min, centrifuged (15 000 g, 5 min), prepared for electrophoresis or stored at −20 ℃. Quantitative analysis of the protein to weight was performed by Bradford method, examined by SDS—PAGE with the amount of 40 μg protein

samples. The target protein were made using 6% separating gel and 4% stacking gel, beta–actin was used as the internal control with 8% separating gel. Transmembrane and blocking, incubated in first antibody and second antibody after rinsing, each sample was repeated for 3 times.

2.8. Indexes and methods

2.8.1. Detection of proliferation and apoptosis of SKOV3

Cell proliferation was measured by MTT cell growth curve: The cells were seeded in 96 plates, $1×10^4$ cells for each hole. There were 6 parallel holes for each cell, added MTT 20 μL/hole after 24 h, incubated for 4 h at 37℃, 5% CO_2. The supernatant were abandoned, then DMSO 150 μL/hole was added. The OD value of each group was detected at the 490 nm wavelength by enzyme immunoassay instrument, the cell growth curve was drawn for 5 d. Apoptosis: Flow cytometry Annexin V–FITC / PI double labeling were analyzed using flow cytometry (BD FACS Calibur). The cells were collected, centrifuged, and washed 3 times with PBS, 10 μL Annexin and FITC were added, gently mixed, 5 μL PI was added. Samples were reacted at room temperature for 15 min avoiding light. The apoptosis proportion of SKOV3 mock transferred to empty vector was used as a control. Annexin V+/PI– stained cells (quadrant LR, low right area) represent early apoptotic cells. Annexin V+/PI+ stained cells (quadrant UR, up right area) represent either late apoptotic cells or necrotic cells.

2.8.2. Assays of cell invasion /migration

24 holes with pore diameter 8 μm and transwell with diameter 6.5 mm were used to mimick a cell interaction model, the bottom of the upper chamber of the membrane were coated with Matrigel in advance to simulate the basement membrane and extracellular matrix. Steps: the cell were starved for 24 h, the Matrigel were melted and put at 4 ℃ refrigerator overnight the day before this experiment. The pipette tip was pre–cooled in ice–cold for 0.5 h during experiment, and the ECM gel was diluted by 1:9 with serum free 1640 (dilute to 1 mg/mL), Matrigel 100 μL was added to each well, the whole process was performed on ice. Then they were placed in an incubator at 37 ℃ for 5 h, thus the Matrigel were from liquid form into gelatum. Hydration of basement membrane: The residual liquid was aspirated from the small chamber, 70 μL serum free 1640 was added in each well at 37 ℃ for 30 min,then the m edium was removed. 10^5/mL SKOV3 cells in logarithmic growth phase was added in each well for 200 μL, 500 μL 1640 medium supplemented with 10% fetal bovine serum were added in the lower chamber. After culturing for 24 h, nutrient solution was abandoned and a cotton swab was used to gently wipe

out the upper layer of transwell. Membrane of transwell was fixed with methanol for 20 min, washed with PBS 3 times, then staining with 0.1% crystal violet for 20 min after airing. For SKOV3 migration ability measurement, the procedure of invasion experimental was the same without Matrigel. After washing, the blue–violet cytoplasm can be clearly observed under microscopic. The invasive cell numbers of 5 fields (upper and lower, left and right, middle) were counted under microscope, the mean value was obtained and the statistical analysis was made.

2.9. Statistical analysis

Data was expressed as mean±SD values, and compared with single factor analysis of variance. Comparisons between groups were tested by LSD, $P<0.05$ were considered to be significant. The data was analyzed with SPSS 10. 0 software (SPSS Inc., Chicago, IN) and GraphPad 5.0. Image J software was used to analysis westernblot semiquantitatively.

3. Results

3.1. siRNA screening results

siRNA screening results were as shown in Table 1. Western blot test found siRNA 005 and 008 sequence 80 nmol/L could significantly inhibit the expression of human SKOV3 cells HE4,and the 80 nmol/L HE4–siRNA had the best interference effect.

3.2. Construction of long–term interference vectors and viral packaging

Double enzyme digestion was conducted for the plasmids, three plasmids could be digested by the *Xho* Ⅰ,but can not be digested by the *Hpa* Ⅰ. The preliminary identification of its function showed the construction was successful, sequencing for the further identification.

3.3. Identification of silencing of HE4

After 3 d of HE4 immunoblotting infected cells, western blot was used to analyze the efficiency of long–term interference. Western blot analysis showed the comparison between the interference group and control group, HE4 protein expression was remarkably decreased (Figure 1).

Sequence 5, 8 had the knockdown effect, and 5 was better. siM was the control cell. Two belts were detected by HE4 antibody, in which the larger strip was highly glycosylated, while the smaller stripe was with lesser extent of

Table 1
siRNA oligonucleotide sequences for the human WFDC2 (HE4) gene.

siRNA		sequence
si1	F	CCGG CAGATGAAATGCTGCCGCAAT CTCGAG ATTGCGGCAGCATTTCATCTG TTTTTG
	R	AATTCAAAAA CAGATGAAATGCTGCCGCAAT CTCGAG ATTGCGGCAGCATTTCATCTG
si 2	F	CCGGGCTGACCAGAACTGCACGCAACTCGAGTTGCGTGCAGTTCTGGTCAGCTTTTG
	R	AATTCAAAAAGCTGACCAGAACTGCACGCAACTCGAGTTGCGTGCAGTTCTGGTCAGC
si 3	F	CCGGGTGTCCTGTGTCACTCCCAATCTCGAG ATTGGGAGTGACACAGGACAC TTTTTG
	R	AATTCAAAAAGTGTCCTGTGTCACTCCCAATCTCGAG ATTGGGAGTGACACAGGACAC
si 4	F	CCGGGCACAGGAGCAGAGAAGACTT CTCGAGAAGTCTTCTCTGCTCCTGTGCTTTTTG
	R	AATTCAAAAAGCACAGGAGCAGAGAAGACTTCTCGAGAAGTCTTCTCTGCTCCTGTGC
si 5	F	CCGGGGACAGCGAATGCGCCGACAA CTCGAGTTGTCGGCGCATTCGCTGTCCTTTTTG
	R	AATTCAAAAAGGACAGCGAATGCGCCGACAACTCGAGTTGTCGGCGCATTCGCTGTCC
si 6	F	CCGGAGTGCGTCTCGGACAGCGAATCTCGAGATTCGCTGTCCGAGACGCACT TTTTG
	R	AATTCAAAAAAGTGCGTCTCGGACAGCGAATCTCGAG ATTCGCTGTCCGAGACGCACT
si 7	F	CCGG GCTCTCTGCCCAATGATAATT CTCGAG AATTATCATTGGGCAGAGAGC TTTTTG
	R	AATTCAAAAAGCTCTCTGCCCAATGATAATT CTCGAG AATTATCATTGGGCAGAGAGC
si 8	F	CCGGCCGCAATGGCTGTGGGAAGGTCTCGAGACCTTCCCACAGCCATTGCGG TTTTTG
	R	AATTCAAAAACCGCAATGGCTGTGGGAAGGTCTCGAG ACCTTCCCACAGCCATTGCGG

glycosylation. From the Figure 1, No 5, 8 si–RNA sequence had the best knockdown effect, so they were selected for the successive test.
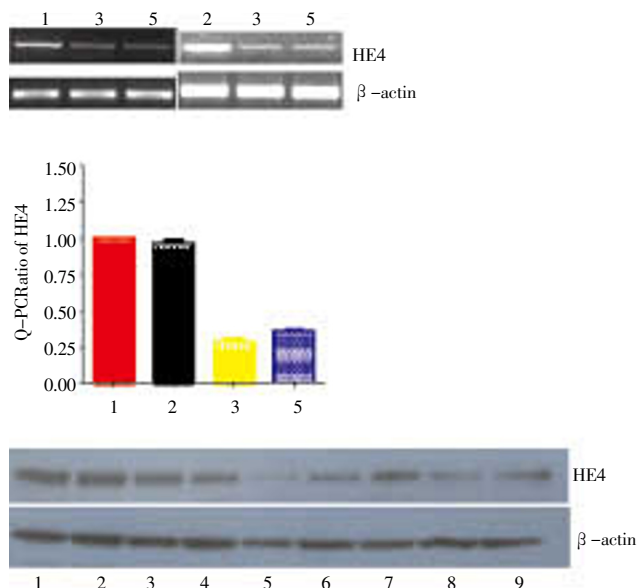

Figure 1. RT – PCR, Q–PCR and Western blot identification of silencing HE4.
1, SKOV3: untreated SKOV3; 2, SKOV3–mock: empty vector SKOV3; 3–9, small interfering strains (SKOV3–HE4–si): SKOV3 transfected HE4 small interfering RNA group.

3.4. Change of the biological behavior of SKOV3 cells after HE4 silencing proliferation

The differences between the knockdown group and controls were statistically significant (P=0.021) (Figure 2). Stable HE4 silencing induced statistically significant inhibition in cell proliferation of SKOV3 (13.42%, 23.13%, 26.21%, 33.23%, 41.21% a at 24–120 h, respectively) for si–HE$_4$5 and (12.26%, 21.13%, 27.28%, 31.32%, 39.89% a at 24–120 h, respectively)

si–HE$_4$8 (Figure 2) (P <0.01). Cells on the third day was determined by proliferation cell nuclear antigen (PCNA) by Western blot (Figure 3). These results were consistent with the MTT results, which indicated that silencing HE4 significantly inhibited ovarian cancer cells growth *in vitro* and HE4 gene got involved in the regulation of proliferation.
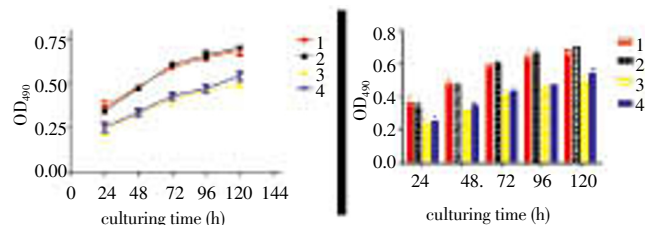

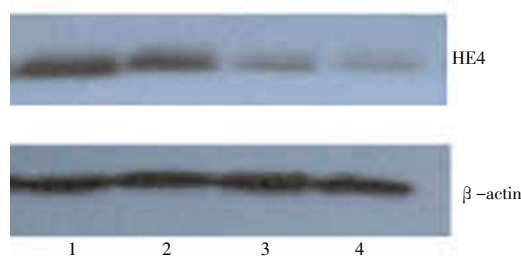**Figure 2.** Cell growth curve after HE4 silencing.

Figure 3. PCNA after HE4 silencing by Western blot.
1, SKOV3: untreated SKOV3; 2, SKOV3–mock: empty vector SKOV3; 3, SKOV3–HE4–si 5: SKOV3 transfected HE4 small interfering RNA group (No. 5); 4, SKOV3–HE4–si 8: SKOV3 transfected HE4 small interfering RNA group (No. 8).

3.5. Apoptosis

Ratio of early apoptotic cells were as follows: (11.91±2.33)% for Si–HE$_4$5 and (9.90±2.31)% for Si–HE$_4$8. As compared to the control: (3.70±1.51)% for untreated SKOV3 group and (6.60 ±0.80)% for SKOV3 empty vector group (P<0.01), suggesting a increasing early apoptosis (Figure 4).

Ratio of late apoptotic cells were: (7.21±2.31)% for Si–HE$_4$5 and (5.42±1.92)% for Si–HE$_4$8. As compared to the control: (6.41±1.23)% for untreated SKOV3 group and (4.78±1.37)% for SKOV3 empty vector group (*P*>0.05), suggesting a similar late apoptosis (Figure 4). Our results indicate that HE4 may mediate early cell apoptosis.
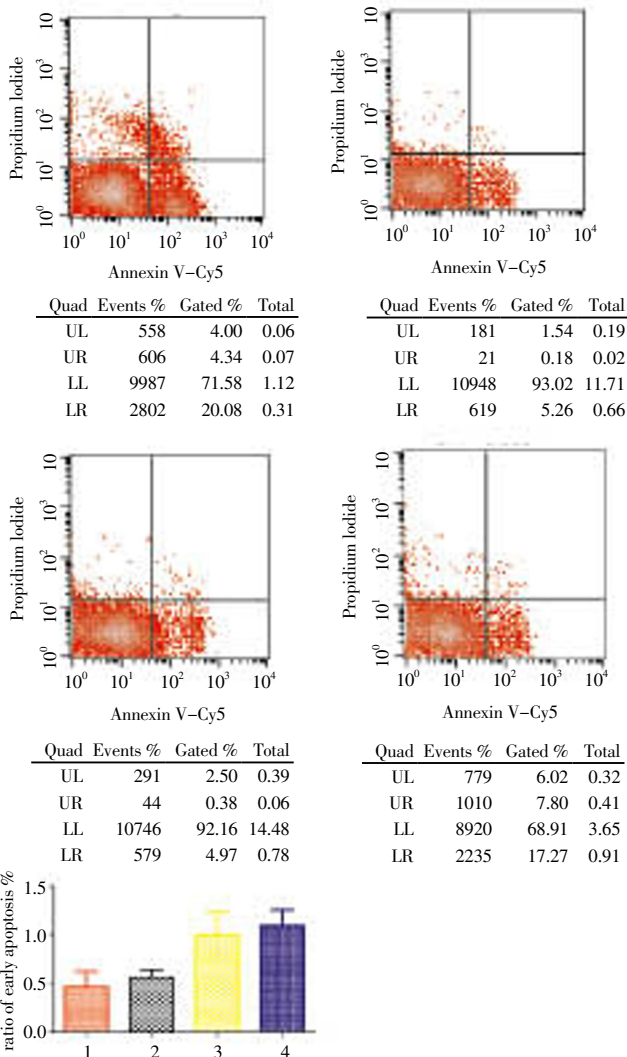

Figure 4. Apoptosis analysis by flow cytometry.
1, Untreated SKOV3 group; 2, SKOV3–mock: SKOV3 empty vector group; 3, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA.(Si. 5); 4, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA (Si. 8).

3.6. Cell cycle

HE4 silencing significantly increased the ratio of cells in G_0/G_1 phase and decreased the number of cells in the S phase in SKOV3.G0/G1 ratio: (63.26±7.94)% for Si–HE$_4$5 and (83.36±9.52)% for Si–HE$_4$8 as compared to the control: (32.41±7.14)% for untreated SKOV3 group and (34.25±8.11)% for SKOV3 empty vector group (*P*<0.01), suggesting a G_0/G_1 arrest of the cell cycle (Figure 5).

S ratio: (29.26±4.34)% for Si–HE$_4$5 and (21.42±5.95)% for Si–HE$_4$8 as compared to the control: (65.41±7.25)% for untreated SKOV3 group and (61.25±6.46)% for SKOV3 empty

vector group (*P*<0.01), suggesting a block in the G1/S phase transition of the cell cycle (Figure 5).

This indicated that HE4 may mediate cell–cycle regulation by promoting the G_0–G_1 phase transition.
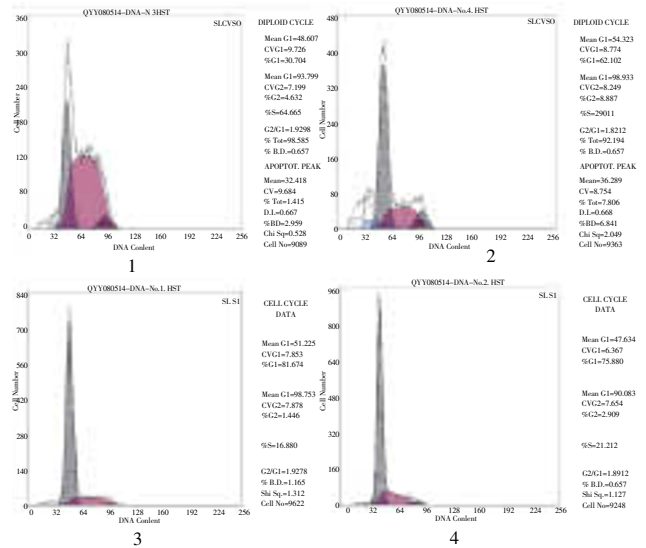

Figure 5. Cell cycle analysis by flow cytometry.
1, Untreated SKOV3 group; 2, SKOV3–mock: SKOV3 empty vector group; 3, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA.(Si. 5); 4, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA (Si. 8).

3.7. Invasion and migration

Invasion test:The cell number of SKOV3 single cultured group which penetrated Matrigel and reached the lower layer of the membrane was (27.0±4.2), the empty vector group were(26.0±4.3), while siRNA–HE4 were (14.0±2.7) and (15.0±3.1), with significant difference between them (*P*<0.01). Multiple comparison showed that the invaded cell numbers in the HE4 low expression group were less than the SKOV3 single cultured group and the SKOV3 empty vector group (*P*<0. 01), no difference was found between the single cultured group and the SKOV3 empty vector group of the invaded cell numbers (*P*>0.05) (Figure 6), which indicated that silencing HE4 lead to impaired capability of invasion of SKOV3 cells.

Migration test: The cell number of SKOV3 single cultured group which penetrated Matrigel and reached the lower layer of the membrane was (37.0±5.1), the empty vector group were (36.0±5.6), while The siRNA–HE4 were (24.0±4.5) and (25.0±5.3) with significant difference between them (*P*<0.01). Multiple comparison showed that the invaded cell numbers in the HE4 low expression group were less than the SKOV3 single cultured group and the SKOV3 empty vector group(*P*<0.01), no difference was found between the single cultured group and the SKOV3 empty vector group of the invaded cell numbers(*P*>0.05) (Figure 7), which indicated that silencing HE4 lead to decreased capability of migration
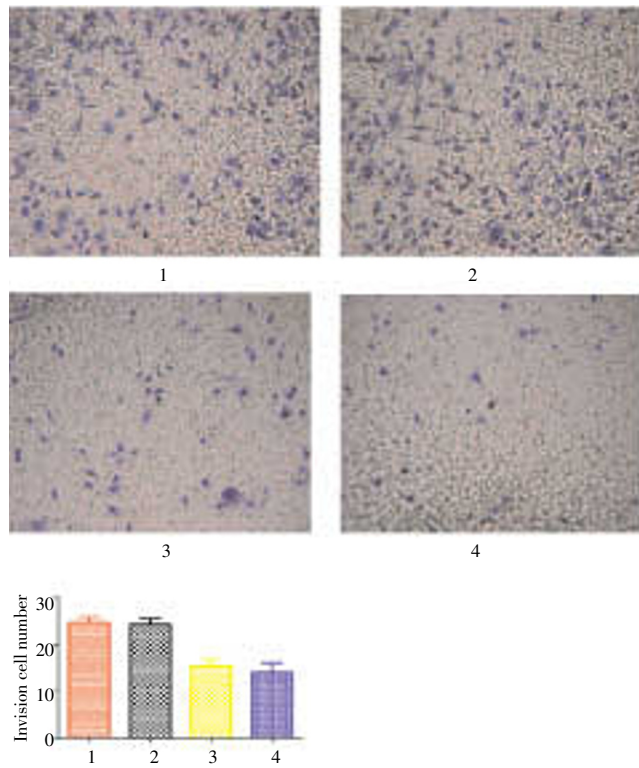
of SKOV3 cells.

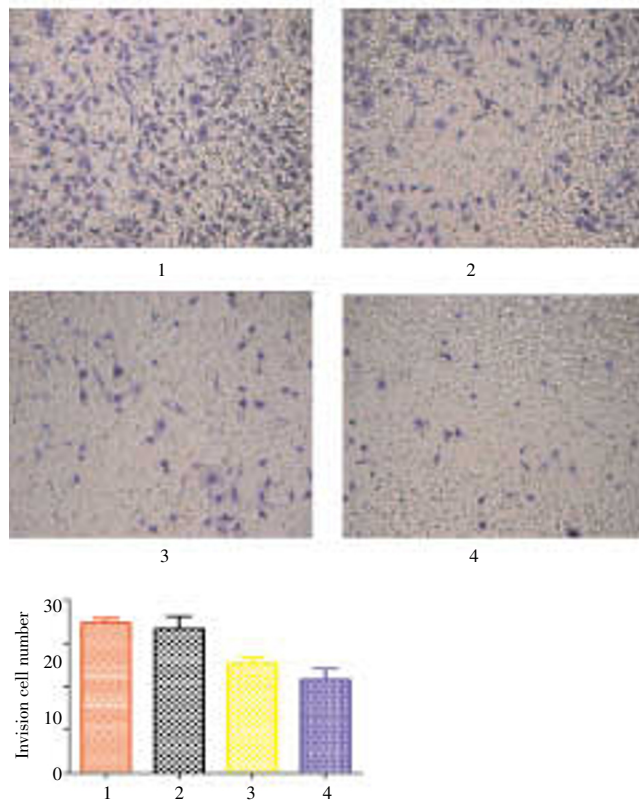**Figure 6.** Cell invasion test (100×).

Figure 7. Cell migration test (100 ×).
1, Untreated SKOV3 group; 2, SKOV3–mock: SKOV3 empty vector group; 3, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA.(Si. 5); 4, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA (Si. 8).

3.8. SKOV3 cell protease expression and ERK pathway protein after HE4 siliencing

We investigated protease which were are correlated in aggressive serous ovarian carcinoma tissue (Matrix metallo proteinases as MMP–9, MMP–2; cathepsin as cathepsin B, C; plasminogen–plasmin system as tPA, uPA), and significant change of protease protein was observed in HE4 transfectants compared with control cells. Silencing HE4 caused marked and significant reduction in MMP–9, MMP–2 and cathepsinB transcription in siHE4 SKOV3 cells, about (38.0±8.2)% decrease in MMP–9, (45.0±7.6)% decrease in MMP–2 and (52.0±8.3)% decrease in cathepsin B (Figure 8), though tPA ,uPA, cathepsin C remained similar compared to control (data not shown).

Proteins involed in ERK pathway as phosphorylated protein as P–EGFR, p– ERK also decreased after HE4 siliencing (Figure 9).
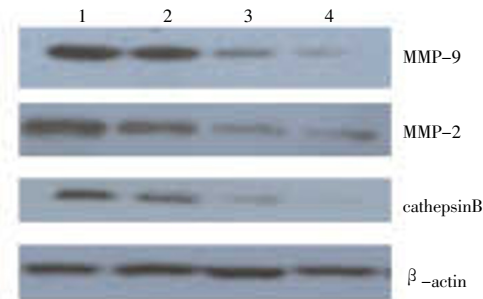

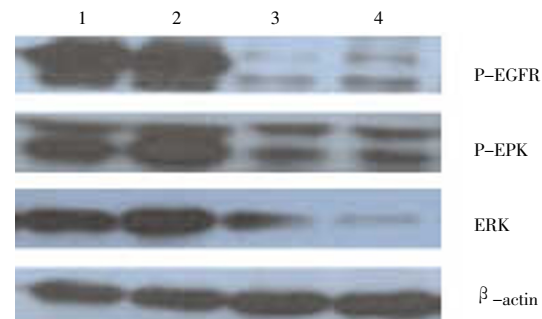**Figure 8.** SKOV3 cell protease expression after HE4 siliencing.

Figure 9. Change of ERK pathway protein after HE4 siliencing.
1, Untreated SKOV3 group; 2, SKOV3–mock: SKOV3 empty vector group; 3, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA (Si. 5); 4, SKOV3–HE4–sh: SKOV3 transfected HE4 small interfering RNA (Si. 8).

4. Discussion

Human epididymal secretory protein 4(HE4) is a new and promising marker for ovarian cancer[2] and it was regarded to be encoded by WFDC2 gene. Results from the OVCAD study found that preoperative HE4 expression in

plasma predicts surgical outcome in primaryovarian cancer patients. hereby, we speculated that HE4 gene might plays an important role in the occurring and developing of the serous ovarian cancer. So clarifying WFDC2 gene function may help us to identify a new therapeutic target in ovarian cancer. Unfortunately, the function of WDC2 gene in ovarian cancer is remained unclarified. In this study, we tried to investigate effect of silencing human WFDC2 (HE4) gene on biological behavior changes as cell proliferation,apoptosis, movement and invasion of human serous ovarian cancer cell line SKOV3 and the possible mechanisms.

After silencing HE4 gene, there were changes on the proliferation, apoptosis, migration and invasion ability of the ovarian carcinoma SKOV3 cell strain, and was accompanied by the change of the relevant cell pathway of ERK. Therefore, we concluded that the HE4 gene was involved in the regulation of ovarian cancer biological behavior, which is consistent with the clinical study of the serum HE4 ovarian cancer prognosis.

In this study, we found that the ability of proliferation of SKOV3 significantly impaired after silencing HE4 gene, suggesting that HE4 involved in the regulation of ovarian serous carcinoma proliferation. This is consistent with the report that WFDC2 genes were involved in the mediation of ovarian cancer proliferation and apoptosis genes[8]. From the structural analogy, the HE4 protein precursor 32 to 73, 76 to 123 amino acids forming two characteristic whey acid protein (whey acidic protein, WAP) domain and it belongs to the disulfide–core (4–DSC) family. Therefore, the HE4 the protein is considered to be the encoding product of the WFDC2 gene, which is a secreted protein characterized by acidic, small single signal peptide and the cysteine–rich polypeptide, is a member in the lactic protein (WAP) domain family.

Epidermal growth factor receptor (epidermalgrowch factor receptor, EGFR)[9] is a transmembrane receptors which gene encoding by C–erbBl and having protein tyrosine kinase (receptor tyrosine kinase RTK) activity. EGFR were composed of three parts, which were extracellular ligand binding domain, transmembrane domain and the intracellular region. The intracellular region is the important parts which can play a key role in tyrosine kinase activity and mediated signal transduction. EGRF widely expressed in human epidermal cells and stromal cells. Studies have been reported in recent years that the EGFR signaling pathway were mostly in closed state of in normal tissues, and is opened after activated in tumor tissue. That was close related to the proliferation of the tumor, the downstream signal transduction pathways of it including Ras–ERK/MAPK, PI3K/AKT and JAK/STAT. Extracellular signal–regulated protein kinase (extracellular–signal regulated protein kinase, ERK) is an important signal transduction protein kinases which introduce extracellular signals by eukaryotic cell into the intracellular reactions, and it regulate a variety of processes including cell growth, differentiation, migration and invasiona. Clinical studies

have shown that there was overexpression of EGFR and ERK in ovarian cancer, and EGRF antibody plays an important role in the treatment of ovarian cancer[10]. In this study, The ERK and EGFR phosphorylation levels were significantly lower after knockdown HE4. This suggests HE4 may inhibit the information of ERK/MAPK downstream signaling pathways of EGFR to inhibit the growth of SKOV3.

Caspase–3 normally exists in cytoplasm in the form of a zymogen (32KD), it is activated in the early stages of apoptosis.The activated Caspase–3 is composed of two large subunits (17KD) and two small subunit (12KD), the corresponding cytoplasm nucleus substrate cleavaged, eventually leading to apoptosis. Caspase–3 activation is often used as an important indicator of apoptosis. Western blot analysis the activation of the Procaspase–3, as well as the cleavage of the substrate poly (ADP–ribose) polymerase [poly (ADP–ribose) polymerase, PARP] by the activated Caspase–3. Western blot detected the cleavage of caspase and the changes of p–Akt expression level. Caspase family plays a very important role in the process of mediating apoptosis, and caspase–3 is a critical execution molecules, which is functional in many pathways of the apoptosis signal transduction.

In our study, although the flow cytometry showed slightly increased early apoptosis ratio in the SKOV3–HE4–si group, 1) There was no difference of late apoptosis ratio between groups; 2)the absolute change of early apoptosis ratio are less than 5%, which is a small probability event; 3)westernblot of Caspase–3 and Bcl–2 proteins which were activated in the early stages of apoptosis failed to show any change between groups(data not shown). Therefore it indicated that the effect of HE4 gene on early apoptosis should be further explored; or it implied that HE4 may affect apoptosis through other means such as the mitochondrial pathway rather traditional Fas/FasL apoptosis pathway.

The study found after silenced HE4 gene, the expression of proteinases (MMP–9, cathepsinB) on SKOV3 cells was reduced, which may explain the change of the invasion force.Proteinases play an important role in the invasion of in tumors including the ovarian cancer[11]. Invasion and metastasis is a direct cause of death for patients with ovarian cancer. By degradation of the extracellular matrix (ECM), matrix metalloproteinases (MMPs) play an important role in the invasion and metastasis of ovarian cancer. Cathepsin is an acidic cysteine proteases exists in lysosomes of all cells, it promote tumor invasion through the degradation of collagen, elastin, laminin, and other components of the extracellular matrix. WFDC2, encodes for a13–kDa protein. Its mature 25–kDa glycosylated form consists of a single peptide and two whey acidic protein (WAP) domains that contain a "four disulfide core" composed of eight cysteine residues. The gene is located on human chromosome 20q12–13.1, a region that includes several genes that encode WAP domain proteins[12]. The WAP architecture domain has an inhibitory ring structure,which can be inserted into

the structural region of the protease activity to inhibit the proteases,and has a certain homology with HE4. human leukocyte protease inhibitor SLPI and lung elastase inhibitor elafin.,members of WAP family including SLPI, Elafin,which characteristics of having a strong protease inhibitor[13]. From point of molecular structure, HE-4, is also a member of WAP family with with two WFDC domains.The WAP architecture domain has an inhibitory ring structure, which can be inserted into the protease activity structure area to inhibiting protease. While HE4 WAP architecture contains two domains, which have some homology with elafin and SLPI, it is a new type of protease inhibitor[14]. Therefore we inferred that HE4 may involved in tumor cell invasion through protease.

　In summary, after silencing HE4 gene, serous ovarian carcinoma SKOV3 cell strain changed on proliferation, apoptosis, migration and invasion ability, and was accompanied by the change of the the relevant mechanism. Therefore, we concluded that the HE4 gene were involved in the regulation of ovarian cancer biological behavior, which is consistent with the clinical study of serum HE4 and ovarian cancer prognosis. HE4 may be a new target for the treatment of ovarian cancer.

Conflict of interest statement

　We declare that we have no conflict of interest.

Acknowledgements

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