의학박사 학위논문

Human epididymis protein 4 (HE4) in chemo-response and prognosis of epithelial ovarian cancer

상피성 난소암에서 human epididymis protein 4 (HE4)의 항암화학요법시 역할과 예후 인자로서의 중요성

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A thesis of the Degree of Doctor of Philosophy

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by

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A thesis submitted in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Medicine

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ABSTRACT

Human epididymis protein 4 (HE4) in chemo-response and prognosis of epithelial ovarian cancer

Objective: Human epididymis protein 4 (HE4) is a novel biomarker for epithelial ovarian cancer. This study evaluated the role of HE4 in chemo-response and prognosis of epithelial ovarian cancer

Materials and methods: We generated HE4 knockdown cells by transfecting with OVCAR-3, and HE4 overexpressing cells by transfecting with SKOV-3. We assessed the effect of HE4 gene silencing and overexpression on the transformed phenotype by examining cell viability assay after exposure to chemotherapeutic agents, invasion, migration, apoptosis and signaling pathway. We studied the expression of HE4 in ovarian cancer tissue and the prognostic significance of this protein. Cytoplasmic staining was graded for intensity and percentage of positive cells. The grades were multiplied to determine an H-score.

Results: HE4 knockdown cells showed increased sensitivity to paclitaxel and

cisplatin. HE4 knockdown inhibited the AKT and Erk pathways. HE4 overexpressing cells showed decreased sensitivity to paclitaxel. HE4 overexpression activated the AKT and Erk pathways. However, neither HE4 knockdown nor overexpression affected invasion, migration and apoptosis. Based on multivariate analysis, the risk of death was significantly higher in patients with an H-score more than 4.

Conclusions: HE4 increases chemoresistance and activates the AKT and Erk pathways. HE4 expression in ovarian cancer tissue is associated with a worse prognosis for epithelial ovarian cancer patients.

Key words: Human epididymis protein 4, ovarian cancer, prognosis, chemotherapy

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Introduction

Ovarian cancer is the sixth most commonly diagnosed cancer worldwide (1). It is the second commonest gynecological cancer, after uterine cervix cancer, in Korea (2). The incidence of ovarian cancer in Korea is similar to worldwide rates, but lower than in Western countries, and is increasing steadily in Korea. Ovarian cancer-related mortality rates as well are increasing in Korea. Age-specific incidence rate and mortality rate increase sharply with advancing age. Ovarian cancer incidence rates are expected to surpass that of uterine cervix cancer in 2015 (3).

Ovarian cancer is clinically unapparent until it is widespread throughout the peritoneal cavity, when the prognosis is dismal. Symptoms are often nonspecific, especially in early-stage cancer. The current standard of care for the treatment of women with ovarian cancer is primary cytoreductive surgery and full surgical staging, followed by platinum-based chemotherapy in the majority of cases.

Unfortunately, more than half of ovarian cancer is diagnosed at advanced stages and the 5-year overall survival is relatively low for FIGO stages III and IV, varying between 23.9% and 37.0% depending on the histologic type (4). The dismal prognosis associated with ovarian cancer is mainly caused by the majority of patients being diagnosed in the advanced stages of the disease. There is thus a need for early detection strategies in order to shift diagnosis to the curable stages of the disease.

Currently, tumor markers for early ovarian cancer with sufficient sensitivity and specificity for screening do not exist (5). CA125 is the most widely used

tumor marker in ovarian cancer; however, its predictive power is far from ideal. It is elevated in about 80% of women with epithelial ovarian cancer but only in 50% of women with early stage disease (6). The specificity of CA125 is limited, since it can be elevated in common benign conditions such as endometriosis and fibroids (7).

Thus research into new biomarkers is active, and human epididymis protein 4 (HE4) is one of the more promising markers. HE4 was first identified by Kirchhoff et al. in epididymal tissue (8) and its gene was found to be expressed primarily in some ovarian cancers (9). Expression of the HE4 gene is highly restricted in normal human tissues, being largely limited to the respiratory epithelium of the proximal airways and the epithelium of the reproductive tracts. It is not expressed in normal ovarian surface epithelium (10). HE4 is overexpressed in ovarian cancer cells and secreted to the sera in the patients with ovarian cancer (11). Other reports show the prognostic value of ovarian cancer and early detection of recurrence (12, 13). The combination of HE4 and CA125 increases the sensitivity and specificity for the detection of ovarian cancer compared to the use of either marker alone. These results utilized the Risk of Ovarian Malignancy Algorithm (ROMA), incorporating both biomarkers to classify patients with adnexal masses as being at low or high risk of having ovarian cancer (14-16).

Despite its clinical utility as a biomarker for ovarian cancer, the role of HE4 in ovarian cancer is not understood. A few studies provide evidence of the cellular and molecular mechanisms that may underlie the motility-promoting role of HE4 in ovarian cancer progression. Expression of HE4 is associated with cancer cell adhesion, migration and tumor growth (17). The HE4 gene is

important in regulating proliferation, apoptosis, migration, invasion of serous ovarian cancer cells (18). HE4 gene silencing induces G0/G1 arrest and blocks the progression from the G1 to S phase. HE4 may be involved in the regulation of the cell cycle, promote ovarian cancer migration and invasion (19), and protect against ovarian cancer progression (20).

Most patients with ovarian cancer receive postoperative chemotherapy. HE4's involvement in the regulation of the cell cycle may affect chemosensitivity and chemoresistance, although this possibility has not been evaluated.

High serum HE4 level is related to poor prognosis and recurrence of ovarian cancer (12, 13, 21-24), but the prognostic significance of HE4 expression in ovarian cancer tissue is not clear. An immunohistochemical analysis of HE4 in paraffin-embedded tissue suggests that HE4 overexpression is not associated with poor prognosis (25). This was not surprising because the proportion of early stage patients in the study population was 83.2%, obviously unrealistic.

Therefore, we evaluated the role of HE4 in ovarian cancer using cell viability assay after exposure to chemotherapeutic agents, motility assay and invasion assay. We additionally studied the expression of HE4 in ovarian cancer tissue and the prognostic significance of this protein.

Materials and Methods

I. HE4 analysis

Cell culture

Human epithelial ovarian cancer cell lines (OVCAR-3 and SKOV-3) were obtained from Korea Cell Line Bank (KCLB) and cultured in RPMI-1640 (Welgene, Daegu, Korea) medium supplemented with 10% heat-inactivated fetal bovine serum (Welgene, Daegu, Korea) and 1% penicillin/streptomycin (Welgene, Daegu, Korea) in a humidified atmosphere containing 5% CO_2 at 37°C.

Lentiviral 293 cells were maintained in RPMI media containing l-glutamine, Fungizone, HEPES, NEAA and 2-mercaptoethanol with 10% FBS in humidified 5% CO_2/95% air atmosphere at 37°C. For transfection, the medium was changed with OPTI-MEM (Invitrogen, Carlsbad, CA) and replaced into DMEM for separation of lentiviral supernatants.

Antibodies and reagents

Rabbit anti-HE4 antibody was purchased from Abcam and anti-phospho-EGFR, phospho-AKT and phospho-extracellular signal-regulated protein kinase (Erk) 1/2 antibodies were obtained from Cell Signaling (Beverly, MA). Mouse anti-Myc and rabbit anti-PARP antibodies were purchased from Sigma Aldrich (St. Louis, MO). Human EGF was purchased from Peprotech (Rocky Hill, NJ).

Generation of HE4 stable cell lines (knockdown cells and overexpressing cells)

For generation of HE4 knockdown cells, lentiviral 293 cells were transfected with packaging plasmids and pLKO-shHE4 construct. The shRNA sequence for HE4 was GCTGACCAGAACTGCACGCAA. The supernatants were harvested 48 hours after transfection, pooled, and passed through a 0.45 μm-pore-size filter. Lentiviral stocks were stored in small aliquots at -80°C for titration and cell infection. OVCAR-3 cells were plated in six-well plates ($5x10^4$ cells per well) and cultured overnight. Lentiviral supernatants were added to 2 ml RPMI containing polybrene (4 μg/ml) and centrifuged for 15 min at 1,500 rpm. Twenty-four hours after final infection, the cells were replaced with fresh RPMI media and the infected cells selected using puromycin (0.5 μg/ml).

To prepare HE4 overexpressing cell lines, SKOV-3 cells were transfected with pcDNA3-6myc-HE4 DNA. Cells were selected by neomycin (0.8 μg/ml) over 14 days. After formation of colonies, a single colony was selected and the expression of HE4 checked with RT-PCR and western blotting.

Cell viability assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in a 96-well flat-bottomed plate ($2x10^3$ cells in 100 μl per well), incubated overnight to allow for cell attachment, and exposed to paclitaxel and cisplatin (Sigma Aldrich, St. Louis, MO) with various doses for 24 hours. 10 μl of CCK-8 solution was added to each well and the cells were incubated for another 2 hours. The viability was measured with a microplate reader at absorbance 450 nm.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured cells using Tri-zol following the manufacturer's protocol (Invitrogen, Carlsbad, CA). The resulting cDNAs were used in PCR using the SYBR-Green master mix (TAKARA, Otsu, Japan) in triplicate. The PCR and data collection were performed using the 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA). All quantitations were normalized to the level of endogenous GAPDH mRNA. The relative quantitation value for each target gene compared to the calibrator for that target is expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing with GAPDH). The relative sample expression levels are presented in a semi-log plot. The following primers were used for qRT-PCR: human HE4 forward CTCCTCAGCCTGCTGCTGTT and reverse GGCACACGCCAGTCTTCTCT; human GAPDH forward ATCTTCTTTTG-CGTCGCCAG and reverse CGTTGACTCCGACCTTCACC.

Immunoblotting

OVCAR-3 and SKOV-3 cells were treated with human EGF and paclitaxel for indicated times and the cells were lysed in a buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA and a protease inhibitor cocktail (GenDEPOT, Barker, TX). For immunoblotting, cell debris was removed by centrifugation and proteins were separated by SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membrane. After incubating with the appropriate primary antibodies, proteins were visualized by chemiluminescence according to manufacturer's instructions

(Pierce, Rockford, IL).

Flow cytometry analysis

After exposure to paclitaxel for 48 hours, cells were harvested by trypsinization. Cell density was adjusted to $1x10^6$ cells per mL in PBS. Apoptosis was determined by staining with Annexin V-FITC and Propidium Iodide (PI) (BD PharMingen, San Diego, CA). Cells were analyzed by flow cytometry on a FACS can using BD FACS diva™ software.

Motility and invasion assay

To investigate transduced cell motility, cells were plated inside Transwell™ inserts (12-mm diameter, 8-lm pore size; Costar, Cambridge, MA) (50,000 cells/well) and allowed to migrate for 8 hours. The cells that migrated to the underside were fixed and stained with hematoxylin and eosin (HE), and the number of cells per microscopic field (x 200) was counted under a microscope. Matrigel was thawed at 4°C overnight, diluted in cold serum-free culture medium, plated onto 24-well plates preloaded with Transwell™ culture inserts, and incubated 5 hours at 37°C. The cells were plated onto the inserts (50,000 cells/well). After 16 hours, the cells on the upper side of the well were removed, and the cells that migrated to the underside were stained and counted under a microscope. All experiments were repeated at least three times.

For the cell scratching (wound healing) assay, a scratch wound was made in the monolayer of cells (grown to 90% confluence) using a cell scraper. The distances of the wounds were then measured using a microscope at 0 and 24 hours.

II. Analysis for prognostic significance of HE4

Case selection

One hundred and sixty-three women were diagnosed with ovarian cancer and treated in the Department of Obstetrics and Gynecology, Gachon University Gil Medical Center from January 2002 to March 2010. Forty-eight patients with non-epithelial ovarian cancer were excluded. One hundred and fifteen women with epithelial ovarian cancer were considered eligible for our study. Formalin-fixed and paraffin-embedded tumor specimens were obtained and all hematoxylin-eosin stained slides of the tumor samples were reviewed.

Tissue Microarray (TMA)

Two representative tumor areas in each ovarian carcinoma were carefully selected and marked on the H&E slides by a circle with a diameter of 3 mm. The corresponding areas of the donor paraffin blocks were punched with a 3 mm hole. These 3 mm-sized tissue cores were transferred and embedded into a recipient block prepared with 24 empty 3 mm-sized holes. Ten TMA blocks (each containing 12 tumor cases) were made. Four μm-thick sections were cut with a microtome and transferred to poly-L-lysine-coated slides.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were placed in citrate buffer (pH 6.0) and heated in a microwave oven for 20 min before application of the rabbit polyclonal antibody to HE4 (1:200 dilution; COVANCE Inc., Princeton, NJ, USA). After incubation with the primary antibody, and addition of the

biotinylated secondary antibody, avidinbiotin immunoperoxidase was applied. Diaminobenzidine was used as the chromogen. Sections were then counterstained with hematoxylin. Tissue sections of human epididymis and normal ovary processed in a comparable manner provided positive and negative controls. Cytoplasmic staining was graded for intensity (0-negative, 1-weak, 2-moderate, and 3-strong) and percentage of positive cells (0, 1 (1–24%), 2 (25–49%), 3 (50–74%), and 4 (75–100%)). The grades for intensity and percentage of positive cells were multiplied to determine an H-score.

III. Statistical analysis

The statistical analyses were performed with IBM SPSS ver. 21.0 (IBM Corp., Armonk, NY, USA). Statistical significance between groups was estimated using the Mann-Whitney U-test. Error bars for all graphs represent standard deviation. The Kaplan-Meier method with the log-rank test and multivariate Cox proportional hazards analysis with hazard ratio (HR) and 95% confidence interval (CI) were used to examine the prognostic significance of HE4. Null hypotheses of no difference were rejected if *P*-values were less than 0.05, or equivalently, if the 95% CIs of risk point estimates excluded 1.

IV. Ethics

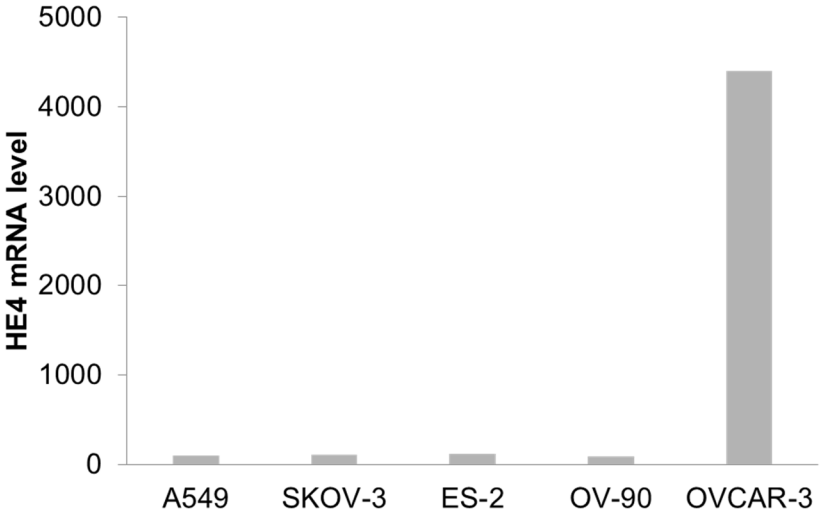
The Institutional Review Board of Gachon University Gil Medical Center, Korea, approved the study (GCIRB2014-161). The informed consent requirement was waived because the current study was performed by a retrospective review.

Results

HE4 mRNA expression in ovarian cancer cell lines

Four ovarian cancer cell lines were screened for expression of HE4 mRNA by qRT-PCR. These cell lines expressed differential basal levels of HE4. The qRT-PCR analysis demonstrated that HE4 mRNA is highly expressed in OVCAR-3 cells and almost undetectable in SKOV-3, ES-2 and OV-90 cells (Figure 1). We used SKOV-3 cell line for generation of HE4 knockdown cells and OVCAR-3 cell line to produce HE4 overexpressing cells.

Figure 1. HE4 mRNA expression in ovarian cancer cell lines. qRT-PCR analysis demonstrated that HE4 mRNA is highly expressed in OVCAR-3 cells and almost undetectable in SKOV3, ES-2 and OV-90 cells.

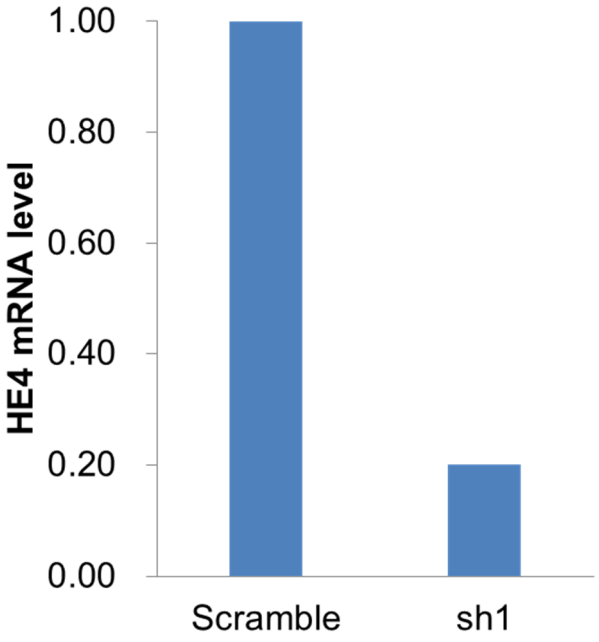


Generation of HE4 knockdown cells

We successfully suppressed HE4 expression in OVCAR-3 cells. The qRT-PCR results demonstrated that shRNA specifically reduced HE4 mRNA of sh1 clone (Figure 2A). Scramble and sh1 clone were compared by western blot analysis. HE4 expression was lower in stably transfected sh1 cells than scramble (Figure 2B).

Figure 2. Generation of HE4 knockdown cells. (A) qRT-PCR identifying HE4 knockdown cell in sh1. (B) Western blot.

A



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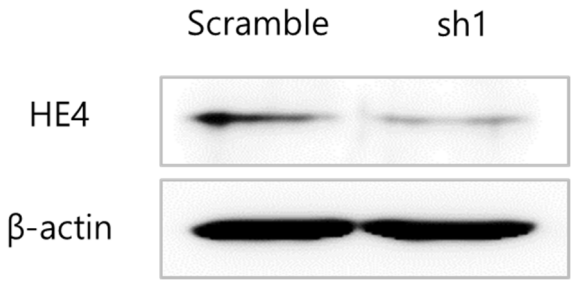


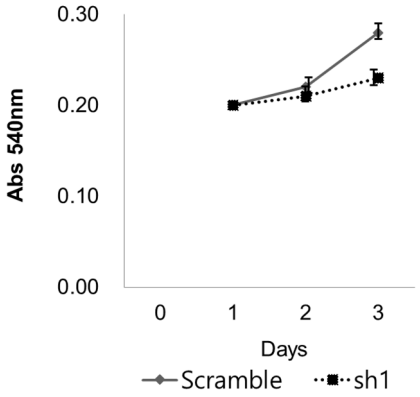
Effect of HE4 knockdown

The effect of HE4 knockdown on ovarian cancer cell growth was analyzed. HE4 knockdown cells grew more slowly. Two days after seeding scramble and sh1 cells, proliferation of scramble was 110.0% and of sh1 cells was 105.0%. There was no significant difference in proliferation between groups ($P = 0.63$). Three days after seeding, proliferation was significantly different between groups ($P = 0.02$). At three days, proliferation of scramble was 140.0% and of sh1 cells was 115.0%. Proliferation of sh1 cells was significantly lower than that of scramble (Figure 3A).

HE4 knockdown increased chemosensitivity to both paclitaxel and cisplatin. Both scramble and sh1 cells showed decreased cell viability showing dose dependent decline to both paclitaxel and cisplatin. The cell viability of sh1 cells was significantly lower than that of scramble at each dose of both paclitaxel and cisplatin (Figure 3B). At 10 nM of paclitaxel, the cell viability of scramble was 93.1% and of sh1 was 80.4% ($P = 0.02$). At 50 nM of paclitaxel, the cell viability of scramble was 95.4% and of sh1 was 63.8% ($P = 0.01$). At 250 nM of paclitaxel, the cell viability of scramble was 80.7% and of sh1 was 56.9% ($P = 0.02$). At 500 nM of paclitaxel, the cell viability of scramble was 71.5% and of sh1 was 52.3% ($P = 0.04$). At 0.5 μM of cisplatin, the cell viability of scramble was 104.6% and of sh1 was 70.9% ($P = 0.04$). At 2 μM of cisplatin, the cell viability of scramble was 79.1% and of sh1 was 46.9% ($P = 0.04$). At 10 μM of cisplatin, the cell viability of scramble was 47.3% and of sh1 was 18.6% ($P = 0.04$). At 50 μM of cisplatin, the cell viability of scramble was 29.2% and of sh1 was 10.2% ($P = 0.03$).

Figure 3. Effect of HE4 knockdown on growth and cell viability. (A) Growth curve. (B) The cell viability assay after exposure to paclitaxel and cisplatin.

A



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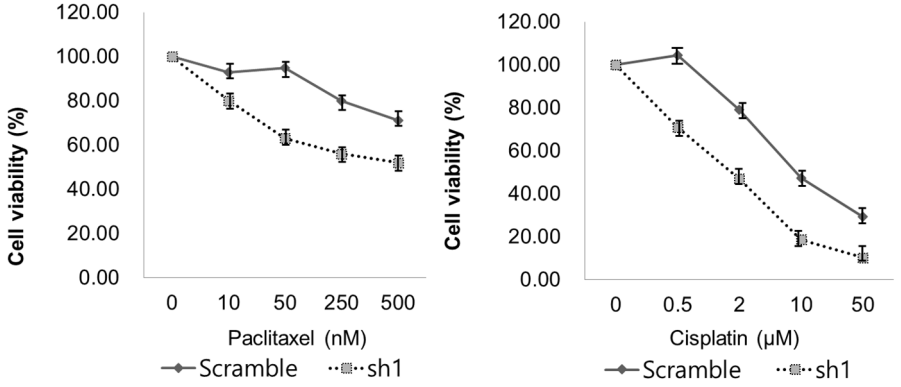

HE4 knockdown had no effect on invasion. The relative invasion rate of sh1 cells was higher than that of scramble (265.3%), but there was no significant difference ($P = 0.10$, Figure 4A).

HE4 knockdown had no effect on migration. The relative migration rate of sh1 was lower than that of scramble (88.7%), but there was no significant difference between scramble and sh1 cells ($P = 0.10$, Figure 4B).

Figure 4. Effect of HE4 knockdown on invasion and migration. (A) Invasion assay. (B) Migration assay.

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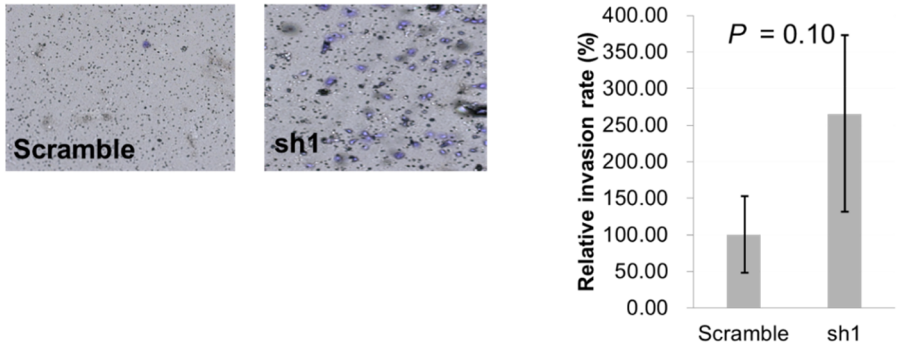

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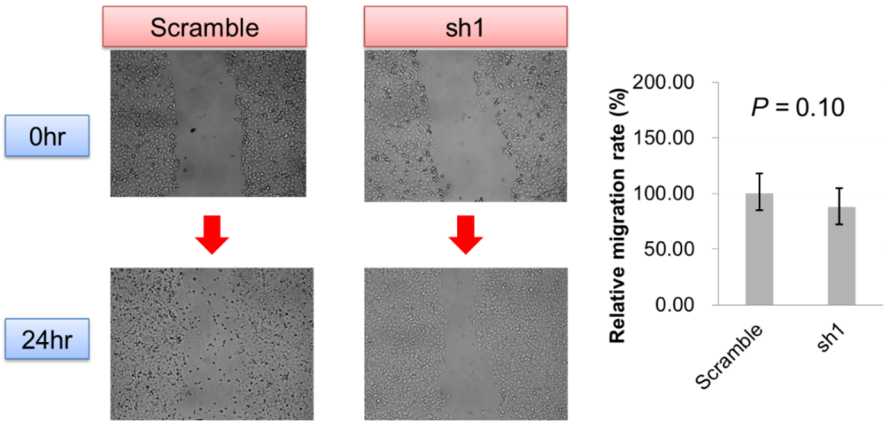
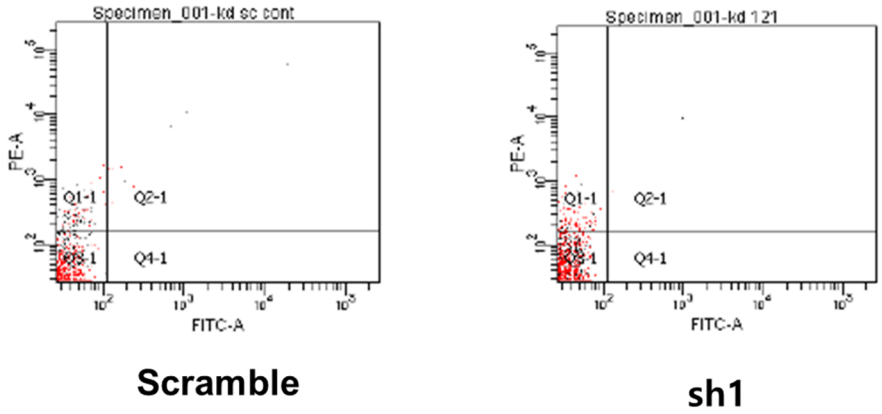


HE4 knockdown had no effect on apoptosis. The ratios of early apoptotic cells were 0.0% for scramble and 0.0% for sh1. The ratios of late apoptotic cells were 0.1% for scramble and 0.0 for sh1 (Figure 5A).

HE4 knockdown inhibited the AKT and Erk pathways. Thirty and 60 minutes after EGF treatment, sh1 cells showed lower AKT expression than scramble. Erk expression was lower in sh1 cells than scramble (Figure 5B).

Figure 5. Effect of HE4 knockdown on apoptosis and signaling pathway. (A) FACS. (B) Change of signaling pathways.

A



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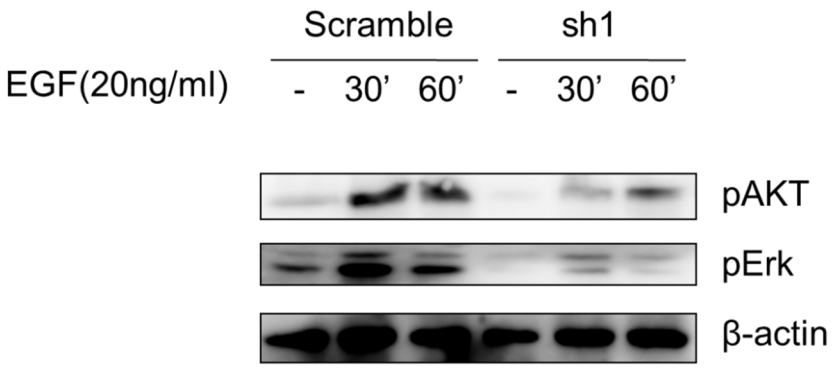

Generation of HE4 overexpressing cell

Generation of HE4 overexpressing cells was successful. We generated pcDNA3-6myc-HE4 DNA and stably transfected SKOV-3 cells. The qRT-PCR analysis (Figure 6A) and western blotting (Figure 6B) showed that expression of HE4 was higher in stably transfected OE4 cells.

Figure 6. Generation of HE4 overexpressing cells. (A) qRT-PCR identifying HE4 overexpression in OE4. (B) Western blotting.

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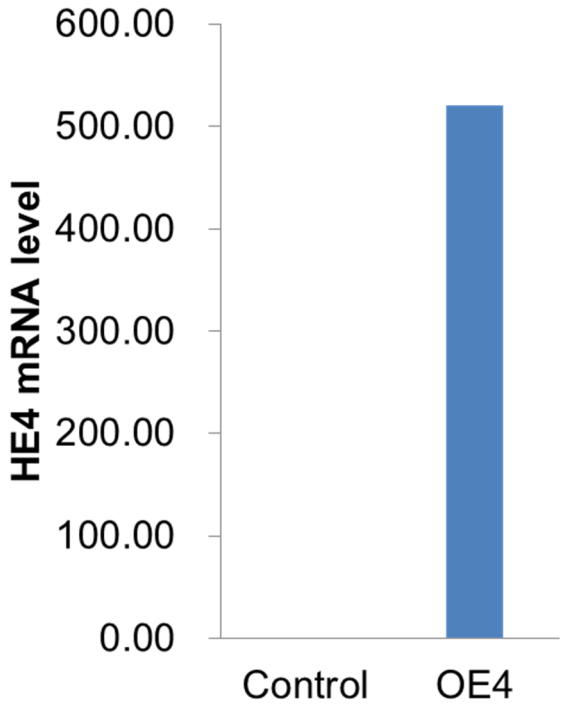

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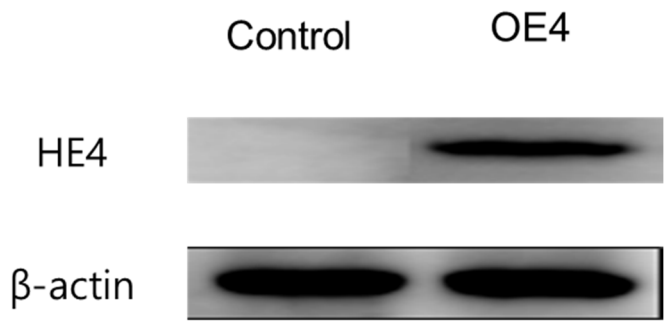

Effect of HE4 overexpression

The effect of HE4 overexpression of ovarian cancer cell growth was analyzed. HE4 overexpressing cells grew at an increased rate. At two days after seeding control and OE4 cells, proliferation control was 105.0% and of OE4 cells was 130.0%. There was a significant difference in proliferation between groups (P = 0.03). Three days after seeding, proliferation was significantly different between groups (P = 0.02), at 125.0% for control and 175.0% for OE4 cells. Proliferation of OE4 cells was significantly higher than that of scramble (Figure 7A).

HE4 overexpression increased chemoresistance to both paclitaxel and cisplatin. Both control and OE4 cells showed decreased cell viability, declining in a dose dependent fashion to both paclitaxel and cisplatin. The cell viability of OE4 cells was significantly lower than that of scramble at each dose of paclitaxel and two doses (0.5 μM and 50 μM) of cisplatin (Figure 7B). At 10 nM of paclitaxel, the cell viability of control was 68.2% and of OE4 was 75.5% (P = 0.02). At 50 nM of paclitaxel, the cell viability of control was 44.5% and of OE4 was 67.9% (P = 0.02). At 250 nM of paclitaxel, the cell viability of control was 45.3% and of OE4 was 67.3% (P = 0.02). At 500 nM of paclitaxel, the cell viability of control was 46.5% and of OE4 was 59.6% (P = 0.03). At 0.5 μM of cisplatin, the cell viability of control was 88.7% and of OE4 was 110.5% (P = 0.03). At 2 μM of cisplatin, the cell viability of control was 95.2% and of OE4 was 120.4% (P = 0.68). At 10 μM of cisplatin, the cell viability of control was 60.5% and of OE4 was 67.4% (P = 0.86). At 50 μM of cisplatin, the cell viability of control was 13.7% and of OE4 was 30.7% (P = 0.02).

Figure 7. Effect of HE4 overexpression on growth and cell viability. (A) Growth curve. (B) The cell viability assay after exposure to paclitaxel and cisplatin.

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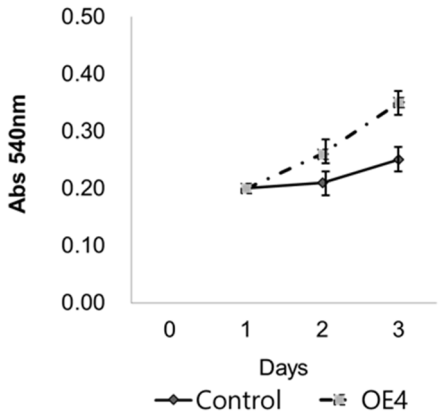

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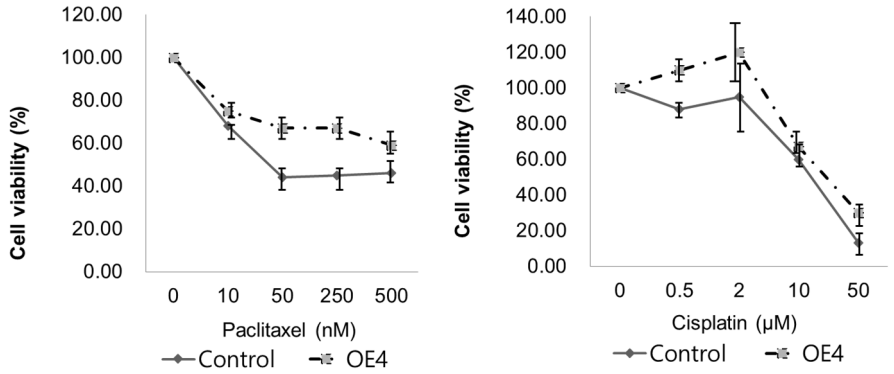
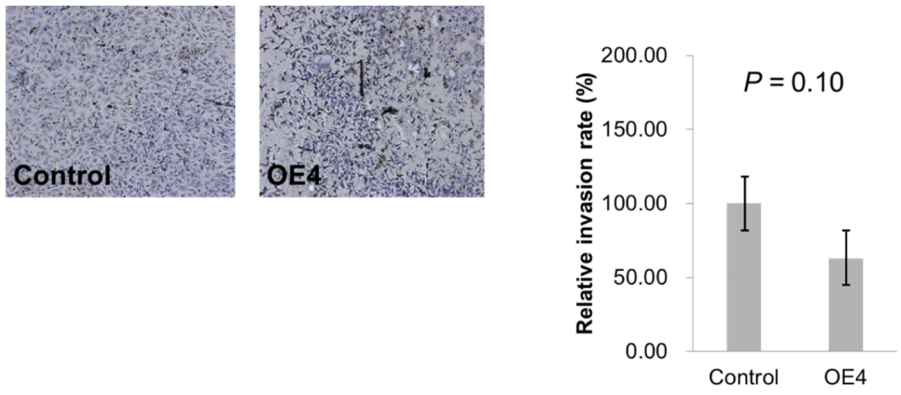


HE4 overexpression had no effect on invasion. The relative invasion rate of OE4 cells was lower than that of control (63.3%), but there was no significant difference ($P = 0.10$, Figure 8A).

HE4 overexpression had no effect on migration. The relative migration rate of OE4 was lower than that of control (98.3%), but there was no significant difference between scramble and sh1 cells ($P = 0.82$, Figure 8B).

Figure 8. Effect of HE4 overexpression on invasion and migration. (A) Invasion assay. (B) Migration assay.

A



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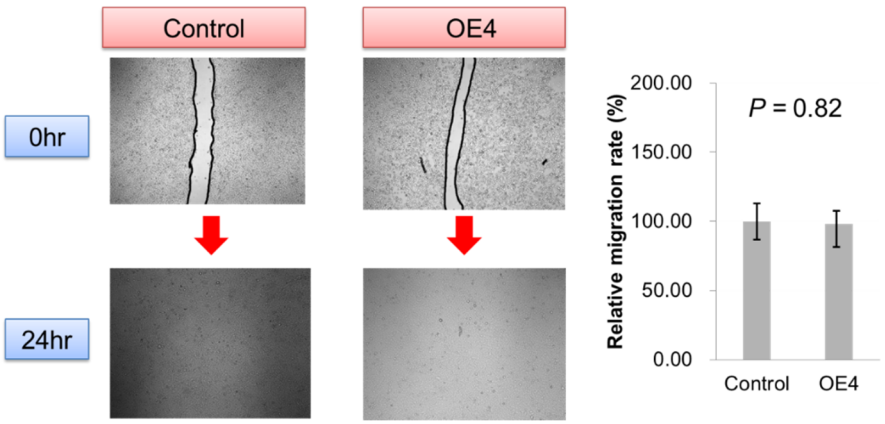


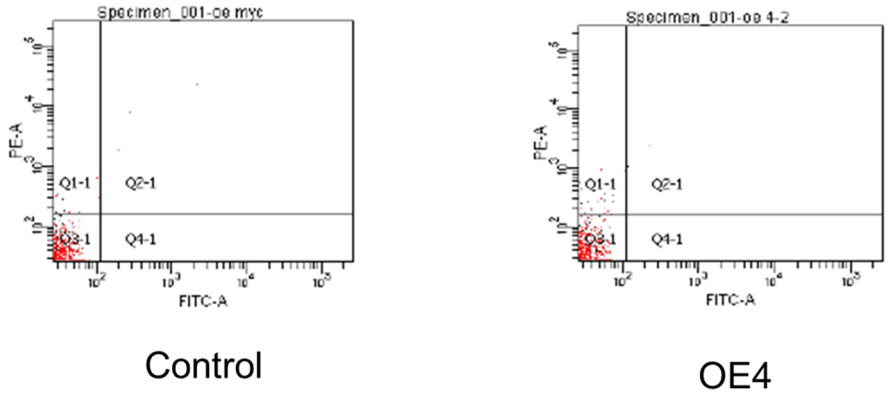
HE4 overexpression had no effect on apoptosis. The ratios of early apoptotic cells were 0.0% for control and 0.0% for OE4. The ratios of late apoptotic cells were 0.0% for control and 0.0 for OE4 (Figure 9A).

HE4 overexpression activated the AKT and Erk pathways. Thirty minutes after EGF treatment, AKT expression of OE4 cell was similar that of control. However, 60 minutes after EGF treatment, OE4 cells showed higher AKT expression than control. Erk expression was higher in OE4 cells than control 60 minutes after EGF treatment (Figure 9B).

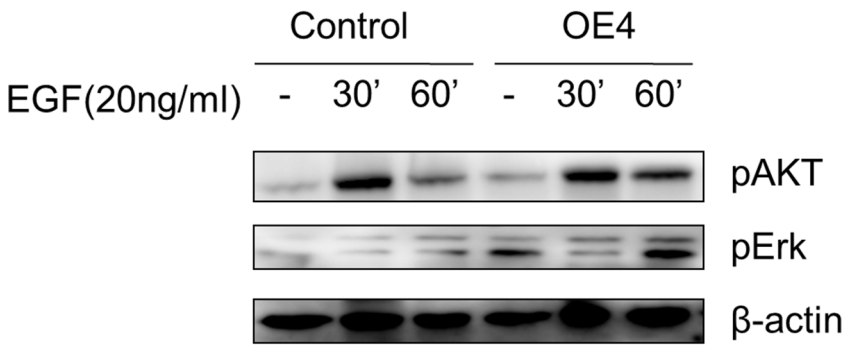
Figure 9. Effect of HE4 overexpression on apoptosis and signaling pathways.

(A) FACS. (B) Change of signaling pathways.

A



B



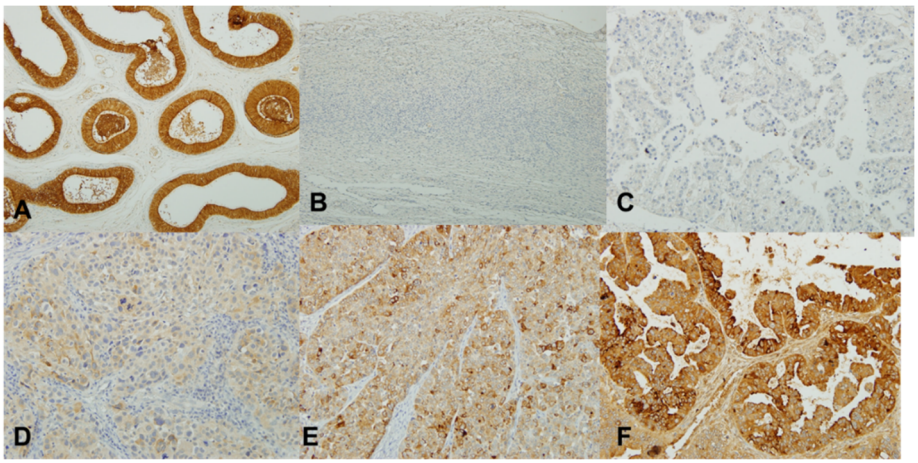
Prognostic significance of HE4

The characteristics of 115 patients are shown in Table 1. Age was categorized into two roughly equal strata: ≤ 50 (48 patients; 41.7%) and > 50 (67patients; 58.3%). With regard to the FIGO stage, 44 cases (38.3%) were stage I, II and 71 cases (61.7%) were stage III, IV. We divided residual disease into two groups: ≤ 1 cm included 69 cases (60.0%) and > 1 cm included 46 cases (40.0%). There were 28 patients (24.3%) with H-score below 4 and 87 patients (75.7%) with more than 4. The initial CA125 level was divided into three groups: < 150 U/mL included 42 cases (36.5%), 150 – 500 U/mL was 42 cases (36.5%) and > 500 was 31 cases (27.0%). The pathologic types were identified as follows: serous (68 cases; 59.1%), clear (19 cases; 16.5%), endometrioid (16 cases; 13.9%) and mucinous (12 cases; 10.5%).

Table 1. Clinical and histological characteristics (n=115)

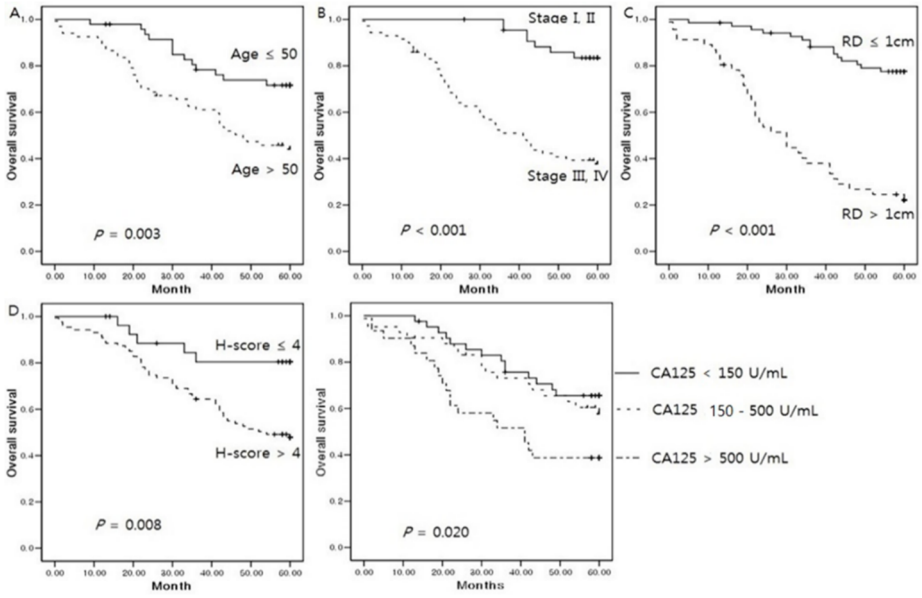
	No (%)
Age	
≤ 50	48 (41.7)
> 50	67 (58.3)
FIGO stage	
I, II	44 (38.3)
III, IV	71 (61.7)
Residual disease	
≤ 1cm	69 (60.0)
> 1cm	46 (40.0)
H score	
≤ 4	28 (24.3)
> 4	87 (75.7)
Preoperative CA125 level (U/mL)	
< 150	42 (36.5)
150 – 500	42 (36.5)
> 500	31 (27.0)
Pathologic type	
Serous	68 (59.1)
Clear	19 (16.5)
Endometrioid	16 (13.9)
Mucinous	12 (10.5)

Figure 10. Immunohistochemistry for HE4. (A) Epididymis as a positive control. (B) No expression of HE4 in normal ovarian tissue. (C) Ovarian cancer showing intensity 0. (D) Ovarian cancer showing intensity 1. (E) Ovarian cancer showing intensity 2. (F) Ovarian cancer showing intensity 3.



The median duration of observation of surviving patients was 58 months (range, 1 to 60 months). Among clinicopathologic factors, age, FIGO stage, residual disease, H-score and initial CA125 were significantly associated with overall survival by univariate analysis (Figure 11).

Figure 11. Univariate analyses of clinicopathologic factors for overall survival. (A) Age, (B) FIGO stage, (C) Residual disease (RD), (D) H-score and (E) Preoperative CA125 level were significantly associated with overall survival.



Based on the multivariate analysis, the risk of death was 2.26 times higher in older patients (HR = 2.26, 95% CI, 1.17 – 4.35) and advanced stage was associated with poor survival (HR = 3.01, 95% CI, 1.01 – 8.83). Residual disease > 1cm (HR = 3.41, 95% CI, 1.53 – 7.59) and H-score > 4 (HR = 3.16, 95% CI, 1.20 – 8.33) were associated with poor survival (Table 2).

Table 2. Cox proportional hazards analyses of prognostic factors for overall survival

Prognostic factors	Hazard ratio	95% confidence interval	*P*-value
Age			0.014
≤ 50	1		
> 50	2.26	1.17 – 4.35	
FIGO stage			0.046
I, II	1		
III, IV	3.01	1.01 – 8.83	
Residual disease			0.003
≤ 1cm	1		
> 1cm	3.41	1.53 – 7.59	
H score			0.020
≤ 4	1		
> 4	3.16	1.20 – 8.33	
Preoperative CA125 level (U/mL)			0.165
< 150	1		
150 - 500	0.45	0.20 – 1.03	
> 500	0.28	0.29 – 1.42	

Discussion

In this study, we examined the role and prognostic significance of HE4 in epithelial ovarian cancer. The biological function of HE4 is not fully understood. Based on its similarity to other whey acidic proteins (WAP), such as secretory leukocyte protease inhibitors (SLPI) Elafin and Eppin, HE4 is hypothesized to be a proteinase inhibitor participating in the host defense function of the lung and in the process of sperm maturation (26).

The mechanism associated with HE4 overexpression in ovarian tumors is unclear, although HE4 does lie in a region of chromosomal amplification often seen in ovarian cancer (27). WAP family members can be induced by pro-inflammatory stimuli during injury, display antiprotease action, and suppress inflammatory proteases. Thus, WAP family members may protect cells or tissues against unregulated proteolytic enzymes during inflammation (28). During ovulation, the ovarian surface epithelium is the site of periodic injury and inflammation, and this inflammatory process is strongly associated with ovarian cancer etiology. The presence of several inflammatory responsive elements including NFκB in the HE4 promoter and the up-regulation of NFκB in ovarian cancer cells (29) further supports the role of inflammation in the upregulation of HE4 in ovarian cancer. Collectively, these observations raise the possibility that inflammatory responsive elements contribute to the high expression of HE4 in ovarian cancer.

The role of HE4 in ovarian carcinoma is unclear. The locus q12-13.1 on chromosome 20 is abnormally amplified in ovarian cancer, so it is speculated that the genes on that region are involved in ovarian carcinogenesis and tumor

progression (30).

A few studies suggested that HE4 knockdown inhibited tumorigenicity of SKOV-3 cell in nude mice (17). In another study, HE4 affected the regulation of the cell cycle (18, 19). We inferred that HE4 might affect chemosensitivity and chemoresistance. HE4 expression in pulmonary adenocarcinoma was of prognostic significance (31), but few studies found prognostic significance of HE4 expression in ovarian cancer.

We examined mRNA expression in four ovarian cancer cell lines. These cell lines expressed differential basal levels of HE4. SKOV-3, ES-2 and OV-90 expressed almost undetectable levels of HE4 mRNA, while OVCAR-3 expressed HE4 mRNA at a high level. We therefore used SKOV-3 for HE4 overexpression and OVCAR-3 for the HE4 knockdown clone. Various ovarian cancer cell lines have been used in previous studies. SKOV-3 was used for overexpression of HE4 (32). SKOV-3 was used for both overexpression and knockdown of HE4 (17). CaoV3 and SKOV-3 was used for overexpression of HE4 (19).

In our study, HE4 knockdown inhibited proliferation of ovarian cancer cells. HE4 knockdown reduced proliferation of SKOV-3(17-19). In our study, HE4 overexpression promoted proliferation of ovarian cancer cell. In a study, HE4 overexpression did not affect the proliferation of SKOV3 cells (17). Contrary to our finding, inhibition of cell proliferation by HE4 overexpression has been reported (20).

We found that HE4 affects chemosensitivity and chemoresistance. Moore et al. recently analyzed the effect of HE4 on chemoresistance. HE4-overexpressing SKOV-3 clones were less sensitive to cisplatin and paclitaxel in

vitro compared to controls. Similarly, HE4-overexpressing OVCAR-8 clones showed increased resistance to cisplatin (32). Both HE4 knockdown and overexpression affected chemosensitivity in the current study.

Most patients with ovarian cancer are treated with postoperative chemotherapy, and chemo-response is an important prognostic factor. The OVCAD study analyzed the association between platinum response and HE4. The plasma and ascites HE4 values were significantly higher in the platinum-non-responder subgroup compared to plasma and ascites values in the platinum sensitive patients (33). Several studies revealed that high serum HE4 level is related to poor prognosis. Considering our study and previous studies, HE4 may worsen the prognosis of ovarian cancer by increasing chemoresistance.

In our study, neither HE4 knockdown nor overexpression had an effect on invasion, although decreased invasion ability by HE4 knockdown has been reported. The invasion and motility abilities of HE4 knockdown cells through the Matrigel were dramatically reduced, 56% compared to control cells (17). Detailed analysis showed fewer invaded cells in the HE4 knockdown group than in the control (18). The stable silencing of HE4 in SKOV-3 cells reduced invaded cells 14%, statistically significant compared to the control (19). However, decreased invasion ability by HE4 overexpression has also been reported. Compared with the controls, the numbers of invading cells in the ES-2 and SKOV-3 colonies transfected with the HE4 gene were reduced by 39.2% and 44.4% (20).

In our study, neither HE4 knockdown nor overexpression affected migration, although inhibition of migration by HE4 knockdown has been reported. HE4 knockdown SKOV3 cells closed 60% of the wounded area by 48 hours. In

contrast, 90% of the wounded control area was closed by 48 hours (17). Multiple comparisons showed that the migrated cell numbers in the HE4 knockdown group were less than the control (18). The stable silencing of HE4 in SKOV-3 cells reduced migrating cells by 27%, significant compared to the control (19). Decreased migration ability by HE4 overexpression nevertheless has been reported, too. Compared with the controls, the numbers of migrating cells in the ES-2 and SKOV-3 colonies transfected with the HE4 gene were reduced by 45.1% and 25.8% (20).

In our study, HE4 had no effect on apoptosis. Research findings differ about the effect of HE4 on apoptosis. HE4 knockdown increased early apoptosis but did not change late apoptosis (18). Conversely, HE4 knockdown slightly induced late apoptosis and slightly decreased early apoptosis in CaoV3 and SKOV-3 cells. Thus, total apoptosis was similar to that observed in the control (19). HE4 overexpression significantly increased the percentage of apoptosis (20).

We examined the effect of HE4 on signaling pathways. AKT and Erk regulate a variety of processes including cell growth, differentiation, migration and invasion. In the current study, HE4 overexpression activated both the AKT and Erk pathways, while HE4 knockdown inhibited both the AKT and Erk pathways. HE4 knockdown impairs Erk phosphorylation (17, 18). So, HE4 seems to affect chemo-response via the AKT and Erk pathways.

HE4 expression was an independent prognostic factor in the current study. We calculated H-score and divided patients into two groups by H-score 4. H-score was used to analyze HE4 expression in normal and malignant human tissues. HE4 immunoreactivity was found in normal glandular epithelium of the female genital tract and breast, the epididymis and vas deferens, respiratory epithelium,

distal renal tubules, colonic mucosa, and salivary glands, consistent with HE4 gene expression. In addition to consistent positivity in ovarian carcinoma, some pulmonary, endometrial, and breast adenocarcinomas, mesotheliomas, and less often, gastrointestinal, renal and transitional cell carcinomas were also positive (34).

HE4 expression was not an independent prognostic factor in one study when dichotomized into negative and positive (25). Problematically, early stage ovarian cancer patients comprised 83.2% of the study sample, not representative of clinical practice populations. Generally, 70% of ovarian cancer patients are diagnosed at an advanced stage. In the current study, 61.7% of patients were diagnosed in advanced stages, concordant with general clinical observations.

Combination chemotherapy with carboplatin and paclitaxel is the treatment of choice after debulking surgery in treating ovarian cancer. Chemoresistance therefore is very important, and many patients experience recurrence due to chemoresistance. Our study found that HE4 enhanced chemoresistance. Thus, we demonstrated that HE4 participates in the process of ovarian cancer cell maturation and enhances the progression of the ovarian cancer. These data suggest that HE4 acts as an ovarian epithelial tumor promoter and is therefore a useful prognostic factor.

In summary, HE4 overexpression aggravated chemoresistance and, especially, HE4 knockdown increased chemosensitivity by activating the AKT and Erk pathways. HE4 expression was associated with worse prognosis and acts as an independent prognostic factor of epithelial ovarian cancer. Our findings are largely in agreement with previous studies. Therapeutic modalities targeting

HE4 for ovarian cancer treatment should be considered.

Conclusion

HE4 increases chemoresistance by activating the AKT and Erk pathways. HE4 expression of ovarian cancer tissue is associated with a worse prognosis of epithelial ovarian cancer.

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국문초록

상피성 난소암에서 human epididymis protein 4 (HE4)의 항암화학요법시 역할과 예후 인자로서의 중요성

연구 목적

Human epididymis protein 4 (HE4)는 상피성 난소암에서 각광받고 있는 새로운 표지자이다. 본 연구는 난소암에서 HE4의 역할과 예후 인자로서의 중요성에 대해서 알아보기 위해 진행되었다.

연구 방법

OVCAR-3 세포주에서 HE4 발현을 감소시켰고, SKOV-3 세포주에서 HE4 발현을 증가시켰다. 각 세포주에서 항암제에 노출 후 세포 생존율, 이동성, 침습성, 세포자멸 및 세포 주기에 영향을 주는 기전에 대해서 비교하였다. 또한, 난소암 조직에서 HE4 발현 정도와 예후와의 관계를 분석하였다. HE에 대한 염색 강도와 HE4 양성 세포 비율로부터 H-score를 결정했다.

결과

HE4 발현이 감소된 세포주에서 paclitaxel과 cisplatin 모두에 대해서 감수성이 증가하였고, AKT와 Erk 경로가 저해되었다. 반면에, HE4 발현이 증가된 세포주에서는 항암제 저항성을 나타냈고, AKT와 Erk 경로가 활성화 되었다. 그러나, HE4는 침습성, 이동성, 세포자멸에 영향을 끼치지 않았다. 다변수 분석에서 H-score가 4 초과하는 환자들에서 사망 위험도가 유의하게 높았다.

결론

HE4는 난소암 세포의 항암제 저항성을 증가시키고, AKT와 Erk 경로를 활성화 시킨다. 난소암 조직에서 HE4 발현이 증가된 환자들의 예후가 좋지 않아, HE4는 난소암 예후 예측에 유의한 인자로 사료된다.

주요어 : Human epididymis protein 4, 난소암, 예후, 항암화학요법

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