

## Original Article

# Midkine as a potential diagnostic marker in epithelial ovarian cancer for cisplatin/paclitaxel combination clinical therapy

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**Abstract:** The paclitaxel/cisplatin combination therapy commonly is used as the first-line treatment for advanced ovarian cancer patients. Midkine (MK), known as a novel tumor biomarker, has been elevated in the serum of patients with epithelial ovarian cancer (EOC). In this study, we aimed to detect the expression of MK in EOC tissues and evaluate clinical value of MK in diagnosis and therapy of EOC. We perform immunohistochemistry analysis to detect MK in EOC sample with postoperative platinum/paclitaxel combination therapy, we found that 71.4% (85 in 119 samples) of these samples were MK positive (> 10% of the cells were stained), and the expression of MK was significantly associated with disease histology ( $P = 0.038$ ) as well as differentiation grade ( $P < 0.001$ ). Moreover, MK positive samples show much more sensitive to cisplatin/paclitaxel combination therapy, compared with MK negative samples ( $P = 0.029$ ). Those results indicated that MK expression might correlate with paclitaxel and/or cisplatin cytotoxicity in clinical therapy of EOC. Then, we evaluated the sensitivity to cisplatin and paclitaxel in 5 ovarian cancer cell lines (ES2, A2870, HO-8910, SKOV3 and SW626), and ES2, the highest MK expression among those cell lines, show the most sensitive to paclitaxel and cisplatin. Further, we confirmed this correlation between MK and paclitaxel and/or cisplatin cytotoxicity with the gain- and lost- of function. Finally, we demonstrated that MK enhanced the cytotoxicity of paclitaxel and/or cisplatin by accumulated cisplatin and paclitaxel through inhibited the expression of multidrug resistance-associated protein 3 (MRP3). In conclusion, MK could be an effective biomarker in diagnosis and therapy of EOC, especially for the drug selection at the time of initial diagnosis.

**Keywords:** Epithelial ovarian cancer (EOC), midkine, cisplatin/paclitaxel combination therapy, drug sensitivity, immunostaining

## Introduction

Epithelial ovarian cancer (EOC) is the fourth most common type of female cancer worldwide [1, 2] and has the highest mortality rate of all gynecologic cancers in China [3]. Debulking surgery is the initial line of therapy for advanced EOC [4], followed with chemotherapy, for which the current standard of care is platinum plus taxane therapy [5]. However, the 5-year survival rate for patients with stage III and IV tumors is less than 40% [6]. This may be attributed to the lack of early detection and appropriate individualized treatment [7-9]. Even patients with similar clinical characteristics, as cancer stage, histological type and grade, exhibit different disease progression and outcomes [10, 11].

Therefore, it is necessary to discover new prognostic factors to support the diagnosis of EOC in patients at a high risk of developing the disease.

In clinical practice, the useful of immunohistochemistry (IHC) panels have been proven in diagnosing ovarian pathology [12, 13]. The distinctive IHC stains of various markers may be used to confirm ovarian tumor categories and distinguish primary or metastatic EOC from colorectal cancer [12, 14]. Single marker or combined markers, as the estrogen receptor (ER) [14], progesterone receptor (PR), antigen KI-67 (Ki67), Wilms tumor protein (WT1), and tumor protein 53 (p53) [15-17], have been reported in diagnosis of gynecologic cancers,

but for EOC diagnosis and therapy, there are no single effective marker which has been reported. Although there are some reports that only demonstrate the proof-of-concept and potential to improve diagnostic efficiency by combining multiple biomarkers, but the role of those kinds of markers have not been understand.

Midkine (MK), as a novel prognostic biomarker in cancer, has been verified overexpressed in many cancers, including ovarian cancer [18-20]. Previous studies have shown that MK is a sensitive and specific tumor marker, and may be used as a novel therapeutic strategy to treat a large number of cancers [21, 22]. Moreover, MK can play a role as a growth factor to support cell survival, and also has been regarded as a drug-resistance factor. Recently, some studies have shown that MK can protect different cancer cells against adriamycin [23], cannabinoid [24], and doxorubicin [22]. However, studies performed by Ota T *et al.* [21] and Kawai H *et al.* [25] show that the downregulation of MK could induce cisplatin resistance in oral squamous cell carcinomas and renal cells, respectively, and a negative correlation between MK expression and cisplatin cytotoxicity was reported in primary lung cancer cells [26]. It might indicate MK demonstrates different roles in different cancer.

Cisplatin/paclitaxel combination therapy, which is known as the first line treatment in a single institutional series of primary untreated advanced ovarian cancer patients, while lots of patients performed low sensitive to this kind therapy. In present study, we evaluated kinds of candidate biomarkers, as WT, MK, ER, PR, Ki67 and p53, and discussed the correlation of those markers with diagnostic performance in EOC. We found that the MK expression show a positive correlation with the predicted the survival time and chemosensitivity of EOC to paclitaxel/cisplatin. Moreover, MK could inhibit the expression of multidrug resistance-associated protein 3 (MRP3), and then enhanced the cytotoxicity of paclitaxel and/or cisplatin by accumulated cisplatin and paclitaxel.

### Methods

#### *Patients*

To identify cases of primary EOC, we examined the surgical pathology files of the Department of Pathology, Nanjing First Hospital (Nanjing,

China) and Jiangsu Cancer Hospital (Nanjing, China) between 2005 and 2012. Diagnosis in each case was based on recommended criteria listed in the classification proposed by the World Health Organization in 2004. All of the patients were Chinese. To select candidate markers, we reviewed two hospital's IHC database results of all patients. The data for 119 primary EOC samples were investigated. The data were analyzed for WT1, P53, Ki67, PR, and ER staining, which are the routine tests used to diagnose EOC at our institution. Informed consent according to the criteria specified by the Nanjing Medical University was obtained from all patients. None of the patients had been treated with preoperative chemotherapy or radiation. The clinic pathologic characteristics of the study population are listed in Supplementary material [Table S1](#). According to standard therapy guidelines, all patients underwent complete surgical resection, and received post-operative platinum/paclitaxel combination therapy. To define chemosensitivity, patients who relapsed 6 months or more after chemotherapy were designated "sensitive", according to the Gynecological Cancer Intergroup (GCG) cancer antigen 125 (CA125) criteria [27].

#### *Serum tumor markers*

Serum levels of traditional tumor markers, CA125, alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA15-3), and carbohydrate antigen 19-9 (CA19-9) were measured by an automated chemiluminescent enzyme immunoassay system (CLEIA).

#### *Immunohistochemistry*

IHC assay was used to determine the expression of MK in EOC tissues. Paraffin-embedded tissues from primary surgery of 119 patients with EOC were used in this assay (The International Federation of Gynecology and Obstetrics [FIGO] stages I-IV). Briefly, 5- $\mu$ m sections of EOC tissue from each case were prepared by extracting the paraffin in xylene. The sections were then re-hydrated and submerged in 3% H<sub>2</sub>O<sub>2</sub> phosphate-buffered saline (PBS) for 15 min to inactivate endogenous peroxidase. The sections were treated with citrate solution (10 mM, pH 6.0; Maixin-Bio, Fujian, China) in a microwave oven at 95-100°C for 10 min to retrieve the antigens. The sections then were incubated with 2% bovine serum albumin for

30 min at room temperature to block nonspecific reactivity. Subsequently, the sections were incubated with antibodies against human MK (Clone ID: EP1143Y; dilution, 1:100; Epitomics, California, USA), WT1 (Clone ID: WT49; Zhongsan-Jingqiao, Beijing, China), p53 (Clone ID: DO7; Zhongsan-Jingqiao, Beijing, China), ER (Clone ID: EP1; Zhongsan-Jingqiao, Beijing, China), PR (Clone ID: EP2; Zhongsan-Jingqiao, Beijing, China), and Ki67 (Clone ID: SP6; Zhongsan-Jingqiao, Beijing, China) overnight at 4°C. After washing 3 times with PBS, an anti-rabbit secondary antibody labeled with horseradish peroxidase (Maixin-Bio, Fujian, China) was added to the sections, and incubated for 1 h at 37°C. The slides were then washed 5 times with PBS, and they were developed using a diaminobenzidine kit (Maixin-Bio, Fujian, China), according to the manufacturer's instructions. Subsequently, the sections were counterstained, dehydrated, and mounted.

### *Cell culture and transfection*

Human ovarian carcinoma cell lines (ES-2, A2780, SW626, HO-8910, and SKOV3) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All the cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, USA) and 100 µg/mL each of streptomycin and penicillin G (Amresco, USA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

The pcDNA3.1/MK recombinant plasmid and a specific RNAi sequence for human MK were transfected using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions (as described previously; 30). Briefly, approximately 5 × 10<sup>4</sup> cells/well were grown overnight in 24-well plates. When the cells reached 60-70% confluence, they were transfected with 0.8 µg of the recombinant plasmid, control plasmid, anti-MK-siRNA (100 nM), or negative control siRNA (100 nM) in serum-free medium using Lipofectamine® 2000. After 4 h of incubation at 37°C, 400 µL RPMI 1640 with 10% FBS was added to the cells. Stable plasmid transfectants were selected in the presence of 0.4 mg/mL G418 (Amersco) during 2 weeks of culture. The cells were then harvested for further testing. The expression of endogenous MK was

analyzed by western blotting and real time polymerase chain reaction, as described in our previous study [28].

MK and MRP3 were analyzed by Q-PCR and Western blot (anti-MK IgG, Clone ID: EP1143Y; dilution, 1:1000, Epitomics, California, USA; anti-MRP3 polyclonal IgG, dilution, 1:2000, Santa Cruz, California, USA) as previously described (24). The primer sequences are as follows: MK, 5-AGCACCGAGGCTTCCT-3 and 5-AGGCTTGCGTCTAGT-3; MRP3 5-CCATCGACTGGAGACT-3 and 5-TATTTGGTGCATTTTCCTCCT-3; GAPDH, 5-TGAAGGTCCGAGTCAACG-3 and 5-CAAAGTTGTCATGGATGA-3.

### *Half of the inhibition concentration (IC<sub>50</sub>)*

The cell counting kit (CCK)-8 was used to monitor the cytotoxicity of cisplatin and paclitaxel. Cells were plated at a density of 2000 cells/well in 96-well plates. After 24 h of culture, the cells were treated with paclitaxel or cisplatin (at final concentrations of 0, 0.625, 1.25, 2.5, 5, 10, or 20 µg/mL) for 48 h. The cells were then treated with the CCK-8 kit reagent (Beyotime, Shanghai, China) according to the manufacturer's instructions and incubated at 37°C for 1.5 h. The absorbance at 570 nm was then estimated using an enzyme calibrator. Inhibition of cell growth was estimated by computing the percentage of viable cells compared with the control. All experiments were carried out in triplicate. The IC<sub>50</sub> was calculated by computing survival curves.

### *Intracellular drug accumulation assay*

The intracellular paclitaxel [29] or cisplatin [21] accumulation assay was performed as described previously. Briefly, cells were cultured in 6-well plates and allowed to adhere to the plate overnight. When the cells reached approximately 80% confluence, they were treated with paclitaxel (4, 2, 1, and 0 µg/mL) or cisplatin (20, 10, 5, and 0 µg/mL) in serum-free medium. After incubation for different time periods, both the dead cells in the culture medium and the adherent viable cells were centrifuged at 1,000 × g for 15 min, harvested, and washed four times with ice-cold PBS.

The harvested samples, which treated with paclitaxel, were incubated with 0.5 mL of ZnSO<sub>4</sub> (4% w/v in H<sub>2</sub>O/CH<sub>3</sub>OH, 70/30) to precipitate proteins, and then the protein sample was dis-

## Cisplatin/paclitaxel sensitization of ovarian cancer by midkine

**Table 1.** Clinicopathological analysis of the expression of various tumor markers in surgically resected EOC

Variables		WT1		MK		ER		PR		Ki67		p53	
		Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Histology	Serous (n = 75)	15	60	16	59	37	38	49	26	35	40	34	41
	Others (n = 44)	20	24	18	26	21	23	27	17	22	22	21	23
	P		0.006		0.038		0.983		0.812		0.872		0.950
FIGO stage	I/II (n = 51)	17	34	14	37	25	26	33	18	24	27	25	26
	III/IV (n = 68)	18	50	20	48	33	35	43	25	33	35	30	38
	P		0.542		0.977		0.895		0.978		0.979		0.730
Grade	G1/G2 (n = 37)	16	21	21	16	19	18	22	15	17	20	19	18
	G3 (n = 82)	19	63	13	69	39	43	54	28	40	42	36	46
	P		0.045		0.000		0.853		0.641		0.000		0.578
Metastasis	Yes (n = 75)	20	55	23	52	27	48	45	30	34	41	31	44
	No (n = 44)	15	29	11	33	31	13	31	13	23	21	24	20
	P		0.516		0.652		0.001		0.343		0.588		0.228
Chemosensitivity	No (n = 39)	13	26	18	21	17	22	23	16	20	19	20	19
	Yes (n = 57)	21	36	13	44	22	35	32	25	30	27	28	29
	P		0.892		0.029		0.781		0.948		0.938		1.000

Legend: ER, estrogen receptor; Ki67, antigen KI-67; MK, midkine; p53, tumor protein 53; PR, progesterone receptor; WT1, Wilms tumor protein; Neg, negative; Pos, positive.

solved with 300  $\mu$ L of CH<sub>3</sub>CN, filtered, and analyzed by high performance liquid chromatography (HPLC) to evaluate the amount of paclitaxel in the cells.

The resulting cells treated with cisplatin were solubilized by direct addition of 1 ml of 70% nitric acid for 10 min at room temperature, and then harvested, diluted to 5 ml by addition of 0.5% nitric acid, and analyzed the amount of cisplatin in the cells via inductively coupled plasma mass spectrometry (ICP-MS).

The protein concentration was estimated using the BCA Protein Assay Kit (Beyotime, Haimen, China), according to the manufacturer's instructions.

### Statistical analysis

The GraphPad Prism5 software (San Diego, CA) was used to analyze the clinical data. The correlations between IHC staining and multiple clinicopathologic characteristics were calculated using Fisher's exact test. Survival was calculated from the day of surgery, and survival curves were obtained using the Kaplan-Meier method. *P*-values of less than 0.05 were considered significant.

## Results

### Profiles of serum tumor markers

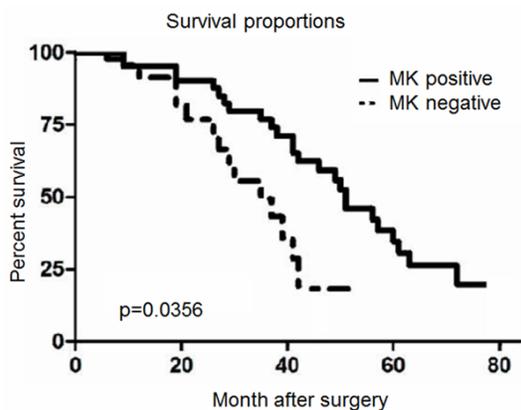
The detection of serum tumor markers was known as an effective method to monitor the

cancer recurrence during the recovery period. According to the clinical characteristics, including age, histology, quantity of ascites, FIGO, histological grade, and metastasis, we grouped 119 primary EOC patients and detected the levels of serum markers, as CA125, AFP, CEA, CA15-3, and CA19-9. As shown in the Supplementary material [Table S2](#), we found that CA-125, a good marker of ovarian cancer [30], is associated with clinical parameters, including histology, quantity of ascites, FIGO and metastasis. AFP, CEA and CA15-3 could also represent some of the parameters. In EOC patients, AFP was associated with the tumor grade, CEA was related with the tumor histology, and CA15-3 levels tended to be positively correlated with increasing ascites quantity in serous EOC. But CA19-9 did not associate with any of the clinical characteristics.

### Correlation between immunohistochemical profiles and clinicopathologic parameters

Previous study revealed that MK might significantly improve the detection efficiency of CA125 for ovarian cancer [19]. To explore the significance of MK in ovarian cancer, we evaluated IHC to analyze the MK expression in 119 EOC sample as well as other tumor biomarkers, as WT, ER, PR, Ki67 and p53. As shown in **Table 1**, we found that the positive samples of WT1, MK, ER, PR, p53 and Ki67 were 70.6%, 71.4%, 51.3%, 36.1%, 53.8% and 52.1% in 119 EOC patients, respectively. The representative imag-

## Cisplatin/paclitaxel sensitization of ovarian cancer by midkine



**Figure 1.** The Kaplan-Meier survival curves computed for the EOC patients with cisplatin/paclitaxel-based chemotherapies. The patients with MK positive expression could significantly survive longer than those with negative expression who all received the cisplatin/paclitaxel-based chemotherapies ( $p = 0.0356$ ).

es were shown in Supplementary material [Figure S1](#). Then we further discussed the relation of WT, MK, ER, PR, Ki67 and p53 with clinicopathologic parameters. In serous ovarian cancer, there were significantly stronger of WT1 ( $P = 0.006$ ) and MK ( $P = 0.038$ ) expression, compared with in other types of ovarian cancer. Moreover, the expression of WT1, MK, and Ki67 were found to be markedly associated with a poor differentiation grade ( $P = 0.045$ ,  $P = 0.000$ , and  $P = 0.000$ , respectively), and ER expression was found to be related with metastasis in EOC ( $P = 0.001$ ). While no correlation was demonstrated between p53 and PR expression or any of the other clinicopathologic parameters examined.

Interestingly, we found that MK positivity patients show more sensitivity to platinum/paclitaxel combination therapy than to single-agent platinum therapy ( $P = 0.029$ ). To investigate the role of MK in chemotherapy, we analyze the correlation between MK expression and outcomes of cisplatin/paclitaxel combination therapy (non-sensitive,  $n = 39$ ; sensitive,  $n = 57$ ). As shown in **Figure 1**, patients with positive and negative MK expression exhibit the significant differences in the clinical outcome and survival rate of cisplatin/paclitaxel-based chemotherapies ( $P = 0.0356$ ). The Kaplan-Meier survival curve demonstrated that the MK positive patients performed a good recovery with cisplatin/paclitaxel-based chemotherapies. Th-

is data indicated that the expression of MK could be an effective index in the clinical used the cisplatin/paclitaxel chemotherapy for EOC patients.

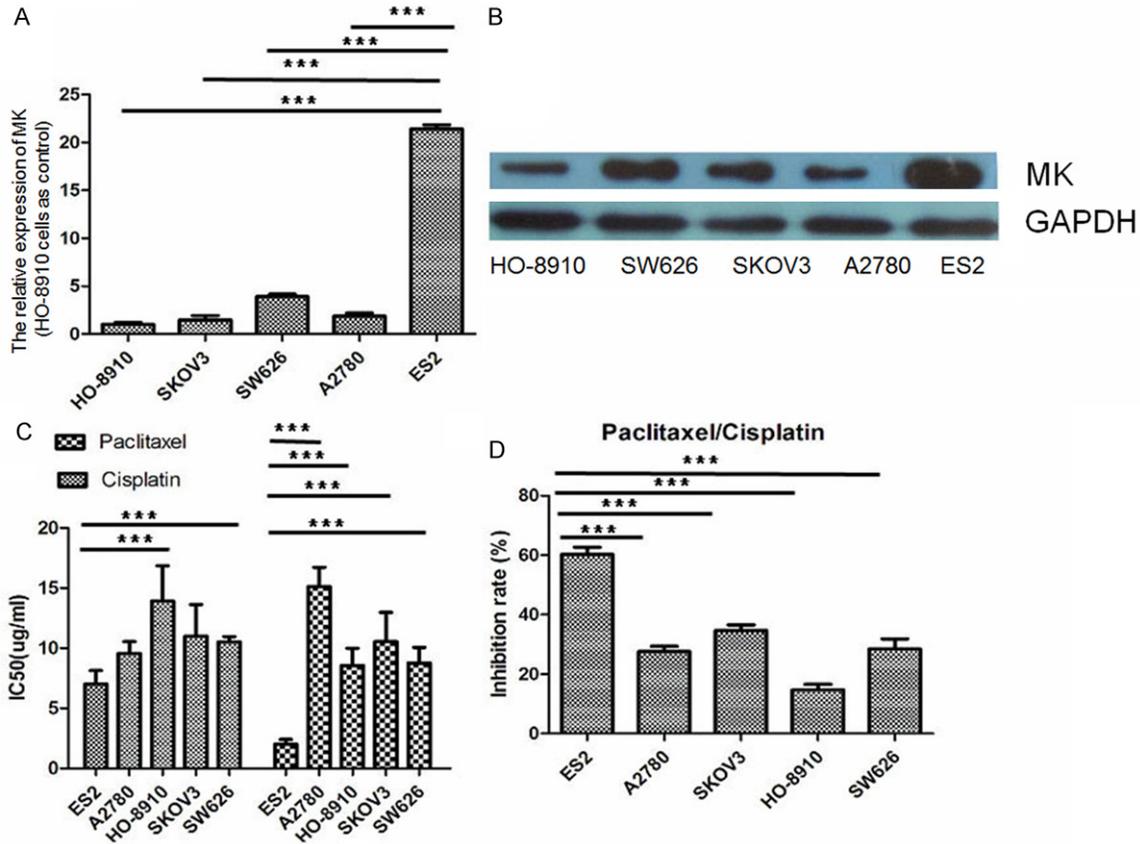
### *MK sensitizes ovarian cancer cells to paclitaxel and cisplatin*

To figure out the role of MK in cisplatin/paclitaxel chemotherapy, we compared the expression of MK and the cytotoxicity of cisplatin and/or paclitaxel in ovarian cancer cell lines, as SKOV3, ES2, SW626, A2780, and HO-8910 cells. As shown in **Figure 2**, those cells show the different expression of MK and exhibited different  $IC_{50}$  values with cisplatin and/or paclitaxel. ES2 cells, which had the highest expression of MK among the 5 cell lines, had the lowest  $IC_{50}$  values (paclitaxel,  $2.02 \mu\text{g}/\text{mL}$ ; cisplatin,  $7.82 \mu\text{g}/\text{mL}$ ). Moreover, combine of cisplatin ( $5 \mu\text{g}/\text{mL}$ ) and paclitaxel ( $2 \mu\text{g}/\text{mL}$ ) to treat, we found that the inhibition of ES2 cells were the highest, compared with other cell lines. Those data suggested that MK expression might contribute to the toxicity of paclitaxel and cisplatin in ovarian cancer cells.

Then, we applied gain- and lost- of function to further evaluate the effect MK effect on paclitaxel and cisplatin sensitivity of ovarian cancer cells, including treated by  $200 \text{ ng}/\text{mL}$  exogenous MK protein, overexpression of MK, or knockdown expression of MK with siRNA. After treated with exogenous MK or overexpressing MK, ES2 cells performed more sensitive to paclitaxel ( $IC_{50}$ ,  $1.43$  and  $0.87 \mu\text{g}/\text{mL}$ , respectively), native ES2 ( $IC_{50}$ ,  $2.02 \mu\text{g}/\text{mL}$ ) or ES2-3.1 ( $IC_{50}$ ,  $2.53 \mu\text{g}/\text{mL}$ ) as the control cells (**Figure 3A** and **3B**). Next, we inhibited MK expression with siRNA. As shown in **Figure 3B** and **3C**, the ES2-MK-siRNA cells performed lower sensitive to paclitaxel, compared with the ES2-Ne-siRNA cells, while the ES2-MK-siRNA cells didn't show lower sensitive to cisplatin, but exogenous MK and overexpressing MK in ES2 cells could enhance the cytotoxicity of cisplatin (**Figure 3B** and **3C**). Those result figured out that MK could strengthen the cytotoxicity of paclitaxel and cisplatin in chemotherapy.

### *MK enhances paclitaxel accumulation in cells*

The paclitaxel plays the cytotoxicity function when it enter into the cells, whether MK regulated the accumulation of paclitaxel. To verify this hypothesis, we quantified intracellular

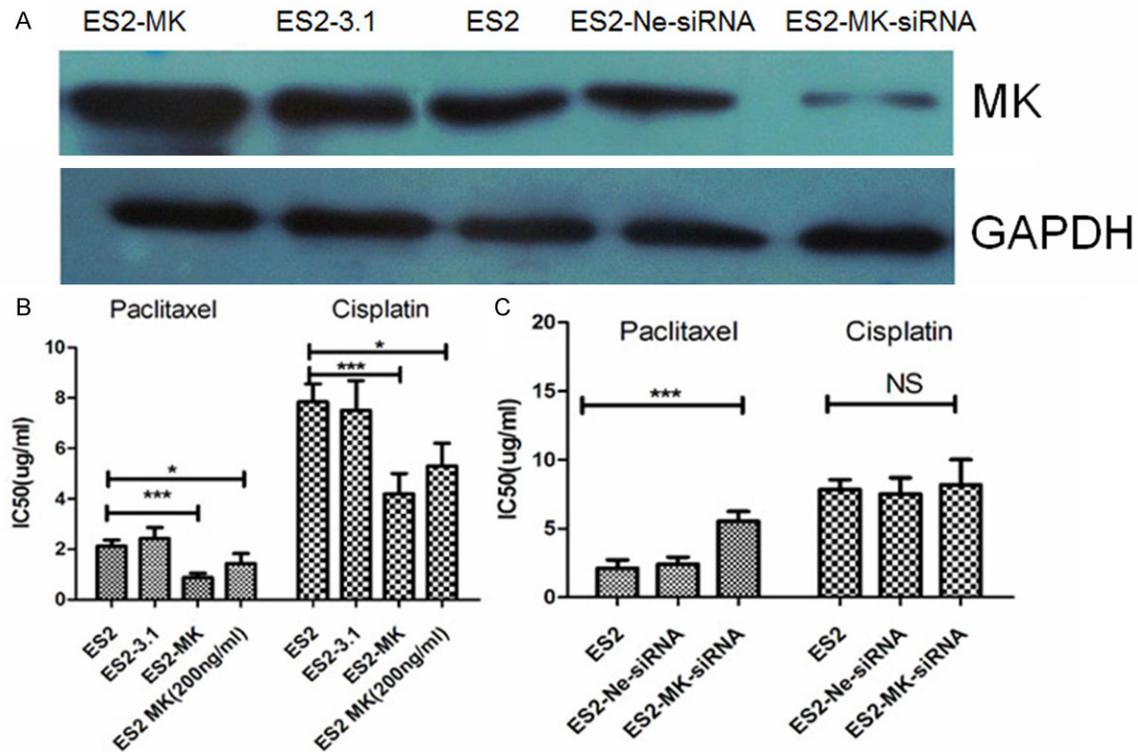


**Figure 2.** Comparison of the expression levels of MK mRNA (A) and protein (B), and the inhibition concentration at 50% (IC<sub>50</sub>) values (C) and inhibition rate of paclitaxel (D) in different human ovarian cancer cell lines: ES2, A2780, HO-8910, Skov3 and SW626. Cells were treated with various concentrations of paclitaxel or cisplatin (0, 0.625, 1.25, 2.5, 5, 10 or 20 μg/mL), and the IC<sub>50</sub> values and the inhibition rates were determined via the cell counting kit (CCK-8) assay. \**P* < 0.05, \*\*\**P* < 0.01 vs. the ES2 group.

paclitaxel during the time course, as 1 h, 2 h, 3 h and 4 h after paclitaxel treated. As shown in **Figure 4**, we found that overexpression of MK could increase the accumulation of paclitaxel, as in ES2-MK cells, while silence the expression of MK would decrease the paclitaxel accumulation, as in ES2-MK-siRNA cells. Moreover, the accumulation of paclitaxel was modified by MK as a dose- and time-dependent manner. ATP-binding cassette, subfamily B, member 1 (ABCB1) was known as an important transporter to help the paclitaxel into the cells. To investigate the mechanisms underlying MK-induced paclitaxel sensitivity, we compared the expression of ABCB1 and the extent of microtubule stabilization by paclitaxel in the ES2, ES2-3.1, and ES2-MK cell lines. However, there was no correlation between the ABCB1, microtubule stabilization, and MK in ES2 cells (data not shown).

#### *MK regulates cisplatin accumulation in cells by MRP3*

MK could not regulate the transporter of paclitaxel, whether MK exhibit the function in the efflux process. The efflux of anticancer agents was mediated by multidrug resistance proteins (MRPs) on cell surface, which is an important mechanism of drug resistance in tumor cells. MRP1 and MRP3 are the major transporters of cisplatin, which could extrude anticancer drugs out of the cell to inhibit the cytotoxicity [31, 32]. After exogenous MK treatment or MK overexpression, we found that MRP3 expression was significantly increased, compared with control cells (**Figure 5A** and **5B**), and the cisplatin accumulation was enhanced in ES2 cells as dose- and time-dependent manner (**Figure 5C** and **5D**). While when we silenced the expression of MK in ES2 cells, the expression of MRP3 and cisplatin accumulation did not show a signifi-



**Figure 3.** The expression levels of MK influenced paclitaxel cytotoxicity in ES2 ovarian cancer cells. Comparison of the expression levels of MK, as measured by western blotting (A) and the IC<sub>50</sub> values of paclitaxel or cisplatin (B, C) in ES2 and ES2-related genetically modified cells. Cells were treated with various concentrations (0, 0.625, 1.25, 2.5, 5, 10, or 20 μg/mL), and the IC<sub>50</sub> values were determined via the CCK-8 assay. \**P* < 0.05, \*\**P* < 0.01 vs. the ES2 group.

cant change (data not shown). Those results might suggest that MK can promote the cisplatin accumulation through inhibited MRP3 expression in ovarian cells.

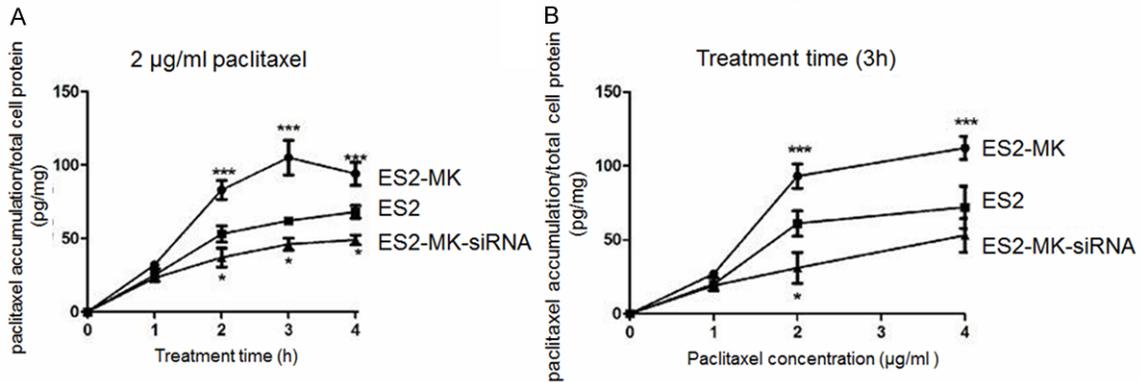
### Discussion

Due to the association between tumor biomarkers and susceptibility of EOC in chemotherapy according to the type of cytotoxic drug used has not been studied in detail, ovarian cancer still has the highest mortality rates among gynecological malignancies [4, 5]. Gregory ER *et al.* reported that the plasma MK was a good biomarker for ovarian cancer, and could significantly improve the diagnostic utility of CA125 in symptomatic women in a multi-analyte panel [19]. Therefore, we analyzed the concentrations of 5 serum biomarkers (CA125, CA19-9, CA15-3, AFP, and CEA) in ovarian cancer patients categorized by clinicopathologic features and 6 histological biomarkers (ER, P53, WT1, PR, Ki67, and MK), and we summarized

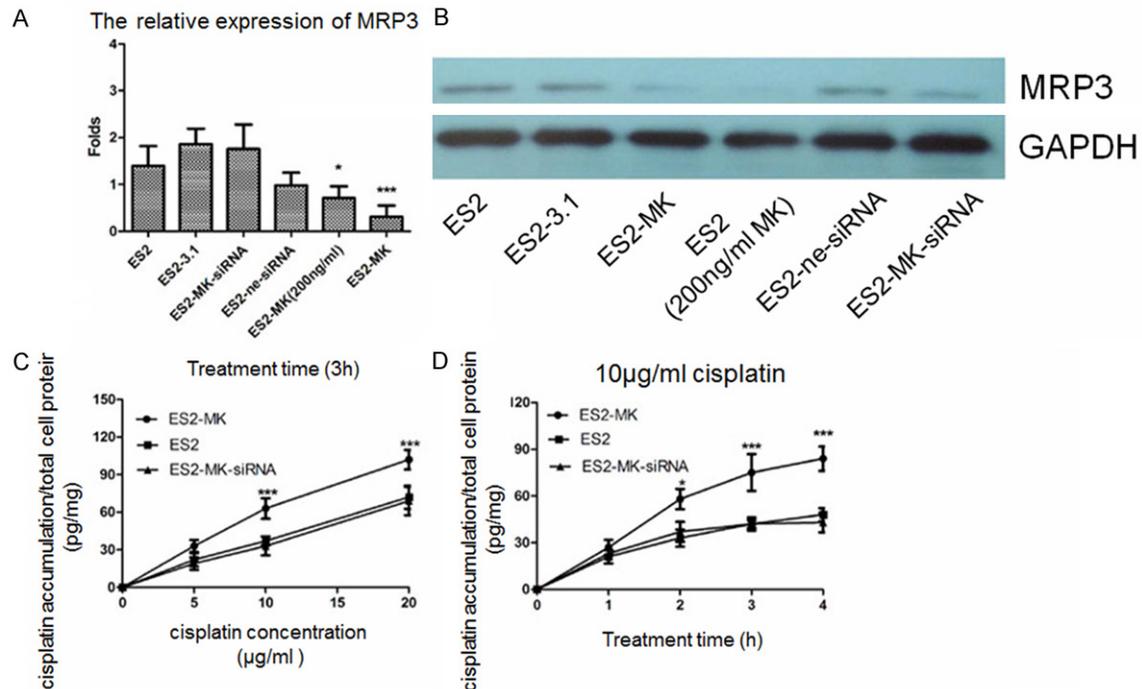
the relationship between serum biomarkers and the histological biomarkers. Our results indicated that MK positivity patients had higher serum levels of CA125 and CEA than those with MK negativity patients. Next, we investigated the prognostic significance of the 6 histological biomarkers and demonstrated a significant correlation between MK expression in tumor tissues and clinicopathologic parameters (stage, grade, and response to platinum/paclitaxel chemotherapy). As reported in previous data, WT1 or Ki67 was more frequently detected in serous ovarian cancer than in other types of ovarian cancer. Taken together, these data support the hypothesis that MK not only plays an important role in tumor progression but may also be an indicator of the response to paclitaxel and/or cisplatin in the clinical treatment of ovarian cancer.

To verify this conclusion from clinical data, 5 ovarian cells with different MK expression levels were performed to detect drug cytotoxicity

## Cisplatin/paclitaxel sensitization of ovarian cancer by midkine



**Figure 4.** MK enhances paclitaxel accumulation in ES2 cells. Paclitaxel accumulation in ES2, ES2-MK, and ES2-MK-siRNA cells. Cells were treated with 2 µg/mL paclitaxel for the indicated times (A) or at the indicated paclitaxel doses for 3 h (B), and paclitaxel accumulation was measured by high performance liquid chromatography. \* $P < 0.05$ ; \*\*\* $P < 0.01$  vs. the ES2 group.



**Figure 5.** MK inhibits the MRP3 expression (A, B) and enhances cisplatin accumulation in ES2 cells (C, D). Cisplatin accumulation in ES2, ES2-MK, and ES2-MK-siRNA cells. Cells were treated with 10 µg/mL paclitaxel for the indicated times (D) or at the indicated cisplatin doses for 3 h (C), and cisplatin accumulation was measured by ICP-MS. \* $P < 0.05$ ; \*\*\* $P < 0.01$  vs. the ES2 group.

*in vitro*. The results revealed that the ES2 cell line, which had the highest expression level of MK, was most sensitive to paclitaxel and/or cisplatin. Subsequently, a similar result was observed for the MK-overexpressing ES2-MK cell line. MK could activate the Akt signal pathway, which is thought to provide intercellular cytoprotective signals to decrease the response to doxorubicin in SK-N-SH cells [33], while in

our study, MK sensitized ovarian cancer cells to paclitaxel and/or cisplatin. The contradictory of these conclusions might indicated that MK regulated the function of drugs in chemotherapy through different mechanism.

The mechanisms underlying the MK-mediated sensitivity to paclitaxel and cisplatin are complex, and might include the induction of cellular

transport disorders, aggravation of detoxification, decline of drug export, as well as alterations in tubulin isotype composition. In our study, ABCB1 expression (which mediates paclitaxel pharmacokinetics) and microtubules quantity were not altered by MK. Consistent with the results of the cytotoxicity assays, accumulation studies also showed that MK significantly increased the intracellular accumulation of paclitaxel in MK-overexpressing cells with a dose dependent manner. Those findings suggest that MK might play an important role in paclitaxel sensitivity, while the mechanism underlying this function of MK is still unclear and needs further research. Interestingly, we discovered that MK could promote the cisplatin accumulation in ES2 cells by inhibiting the MRP3 expression.

Our study has some limitations. Even though our samples were collected from patients with ovarian cancers, all patients had epithelial ovarian cancers. Therefore, the relationship between MK and paclitaxel/cisplatin sensitivity identified in this study might be limited to this tumor type. The association between MK and sensitivity to ovarian cancer needs to be validated in a larger sample size. Moreover, we could not completely eliminate interference caused by surgical debulking, which is a sub-optimal surgery and could lead to residual tumor cells and affect recurrence and metastasis in our study.

In conclusion, our data suggest that MK may be a useful biomarker for ovarian cancer and the expression of MK in tumor tissues may help to predict the aggressiveness of the cancer and sensitivity to paclitaxel/cisplatin chemotherapy. However, larger cohort studies are needed to confirm these findings. The association between MK expression and paclitaxel and cisplatin accumulation in tumor cells is an important finding that needs to be considered during drug selection at the time of initial diagnosis.

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### Disclosure of conflict of interest

The authors do not report any conflict of interest regarding this work.

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### References

- [1] Konstantinopoulos PA and Matulonis UA. Current status and evolution of preclinical drug development models of epithelial ovarian cancer. *Front Oncol* 2013; 3: 296.
- [2] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T and Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008; 58: 71-96.
- [3] Zhu P, Ning Y, Yao L, Chen M and Xu C. The proliferation, apoptosis, invasion of endothelial-like epithelial ovarian cancer cells induced by hypoxia. *J Exp Clin Cancer Res* 2010; 29: 124.
- [4] Rajanbabu A, Kuriakose S, Ahmad SZ, Khadakban T, Khadakban D, Venkatesan R and Vijaykumar DK. Evolution of surgery in advanced epithelial ovarian cancer in a dedicated gynaecologic oncology unit-seven year audit from a tertiary care centre in a developing country. *Ecancermedicallscience* 2014; 8: 422.
- [5] Jayson GC, Kohn EC, Kitchener HC and Ledermann JA. Ovarian cancer. *Lancet* 2014; 384: 1376-1388.
- [6] Tomao F, Papa A, Rossi L, Strudel M, Vici P, Lo Russo G and Tomao S. Emerging role of cancer stem cells in the biology and treatment of ovarian cancer: basic knowledge and therapeutic possibilities for an innovative approach. *J Exp Clin Cancer Res* 2013; 32: 48.
- [7] Bast RC Jr. Molecular approaches to personalizing management of ovarian cancer. *Ann Oncol* 2011; 22 Suppl 8: viii5-viii15.
- [8] Moore RG and Maclaughlan S. Current clinical use of biomarkers for epithelial ovarian cancer. *Curr Opin Oncol* 2010; 22: 492-497.
- [9] Bast RC Jr. Early detection of ovarian cancer: new technologies in pursuit of a disease that is neither common nor rare. *Trans Am Clin Climatol Assoc* 2004; 115: 233-247; discussion 247-238.
- [10] Romero-Laorden N, Olmos D, Fehm T, Garcia-Donas J and Diaz-Padilla I. Circulating and disseminated tumor cells in ovarian cancer: a systematic review. *Gynecol Oncol* 2014; 133: 632-639.
- [11] Musrap N and Diamandis EP. Revisiting the complexity of the ovarian cancer microenviron-

- ment-clinical implications for treatment strategies. *Mol Cancer Res* 2012; 10: 1254-1264.
- [12] Park SY, Kim BH, Kim JH, Lee S and Kang GH. Panels of immunohistochemical markers help determine primary sites of metastatic adenocarcinoma. *Arch Pathol Lab Med* 2007; 131: 1561-1567.
- [13] Davidson B, Smith Y, Nesland JM, Kaern J, Reich R and Trope CG. Defining a prognostic marker panel for patients with ovarian serous carcinoma effusion. *Hum Pathol* 2013; 44: 2449-2460.
- [14] De Stefano I, Zannoni GF, Prisco MG, Fagotti A, Tortorella L, Vizzielli G, Mencaglia L, Scambia G and Gallo D. Cytoplasmic expression of estrogen receptor beta (ERbeta) predicts poor clinical outcome in advanced serous ovarian cancer. *Gynecol Oncol* 2011; 122: 573-579.
- [15] Piek JM, van Diest PJ, Zweemer RP, Jansen JW, Poort-Keesom RJ, Menko FH, Gille JJ, Jongma AP, Pals G, Kenemans P and Verheijen RH. Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. *J Pathol* 2001; 195: 451-456.
- [16] Netinatsunthorn W, Hanprasertpong J, Dech-sukhum C, Leetanaporn R and Geater A. WT1 gene expression as a prognostic marker in advanced serous epithelial ovarian carcinoma: an immunohistochemical study. *BMC Cancer* 2006; 6: 90.
- [17] Lamy PJ, Pujol P, Thezenas S, Kramar A, Rouanet P, Guilleux F and Grenier J. Progesterone receptor quantification as a strong prognostic determinant in postmenopausal breast cancer women under tamoxifen therapy. *Breast Cancer Res Treat* 2002; 76: 65-71.
- [18] Jones DR. Measuring midkine: the utility of midkine as a biomarker in cancer and other diseases. *Br J Pharmacol* 2014; 171: 2925-2939.
- [19] Rice GE, Edgell TA and Autelitano DJ. Evaluation of midkine and anterior gradient 2 in a multimarker panel for the detection of ovarian cancer. *J Exp Clin Cancer Res* 2010; 29: 62.
- [20] Nakanishi T, Kadomatsu K, Okamoto T, Tomoda Y and Muramatsu T. Expression of midkine and pleiotropin in ovarian tumors. *Obstet Gynecol* 1997; 90: 285-290.
- [21] Ota T, Jono H, Ota K, Shinriki S, Ueda M, Sueyoshi T, Nakatani K, Hiraishi Y, Wada T, Fujita S, Obayashi K, Shinohara M and Ando Y. Down-regulation of midkine induces cisplatin resistance in human oral squamous cell carcinoma. *Oncol Rep* 2012; 27: 1674-1680.
- [22] Chu F, Naiditch JA, Clark S, Qiu YY, Zheng X, Lautz TB, Holub JL, Chou PM, Czurylo M and Madonna MB. Midkine Mediates Intercellular Crosstalk between Drug-Resistant and Drug-Sensitive Neuroblastoma Cells In Vitro and In Vivo. *ISRN Oncol* 2013; 2013: 518637.
- [23] Xu YY, Mao XY, Song YX, Zhao F, Wang ZN, Zhang WX, Xu HM and Jin F. Midkine confers Adriamycin resistance in human gastric cancer cells. *Tumour Biol* 2012; 33: 1543-1548.
- [24] Lorente M, Torres S, Salazar M, Carracedo A, Hernandez-Tiedra S, Rodriguez-Fornes F, Garcia-Taboada E, Melendez B, Mollejo M, Campos-Martin Y, Lakatos SA, Barcia J, Guzman M and Velasco G. Stimulation of the midkine/ALK axis renders glioma cells resistant to cannabinoid antitumoral action. *Cell Death Differ* 2011; 18: 959-973.
- [25] Kawai H, Sato W, Yuzawa Y, Kosugi T, Matsuo S, Takei Y, Kadomatsu K and Muramatsu T. Lack of the growth factor midkine enhances survival against cisplatin-induced renal damage. *Am J Pathol* 2004; 165: 1603-1612.
- [26] Lv M, Mou Y, Wang P, Chen Y, Wang T and Hou Y. Diagnostic and predictive role of cell-free midkine in malignant pleural effusions. *J Cancer Res Clin Oncol* 2013; 139: 543-549.
- [27] Rustin GJ, Quinn M, Thigpen T, du Bois A, Pujade-Lauraine E, Jakobsen A, Eisenhauer E, Sagae S, Greven K, Vergote I, Cervantes A and Vermorken J. Re: New guidelines to evaluate the response to treatment in solid tumors (ovarian cancer). *J Natl Cancer Inst* 2004; 96: 487-488.
- [28] Zhao S, Wang H, Nie Y, Mi Q, Chen X and Hou Y. Midkine upregulates MICA/B expression in human gastric cancer cells and decreases natural killer cell cytotoxicity. *Cancer Immunol Immunother* 2012; 61: 1745-1753.
- [29] Vicari L, Musumeci T, Giannone I, Adamo L, Conticello C, De Maria R, Pignatello R, Puglisi G and Gulisano M. Paclitaxel loading in PLGA nanospheres affected the in vitro drug cell accumulation and antiproliferative activity. *BMC Cancer* 2008; 8: 212.
- [30] Diaz-Padilla I, Razak AR, Minig L, Bernardini MQ and Maria Del Campo J. Prognostic and predictive value of CA-125 in the primary treatment of epithelial ovarian cancer: potentials and pitfalls. *Clin Transl Oncol* 2012; 14: 15-20.
- [31] Lin S, Hoffmann K, Xiao Z, Jin N, Galli U, Mohr E, Buchler MW and Schemmer P. MEK inhibition induced downregulation of MRP1 and MRP3 expression in experimental hepatocellular carcinoma. *Cancer Cell Int* 2013; 13: 3.
- [32] Kuan CT, Wakiya K, Herndon JE 2nd, Lipp ES, Pegram CN, Riggins GJ, Rasheed A, Szafranski SE, McLendon RE, Wikstrand CJ and Bigner DD. MRP3: a molecular target for human glioblastoma multiforme immunotherapy. *BMC Cancer* 2010; 10: 468.
- [33] Rebbaa A, Chou PM and Mirkin BL. Factors secreted by human neuroblastoma mediated doxorubicin resistance by activating STAT3 and inhibiting apoptosis. *Mol Med* 2001; 7: 393-400.

## Cisplatin/paclitaxel sensitization of ovarian cancer by midkine

**Table S1.** Demographics and clinical characteristics of the study population

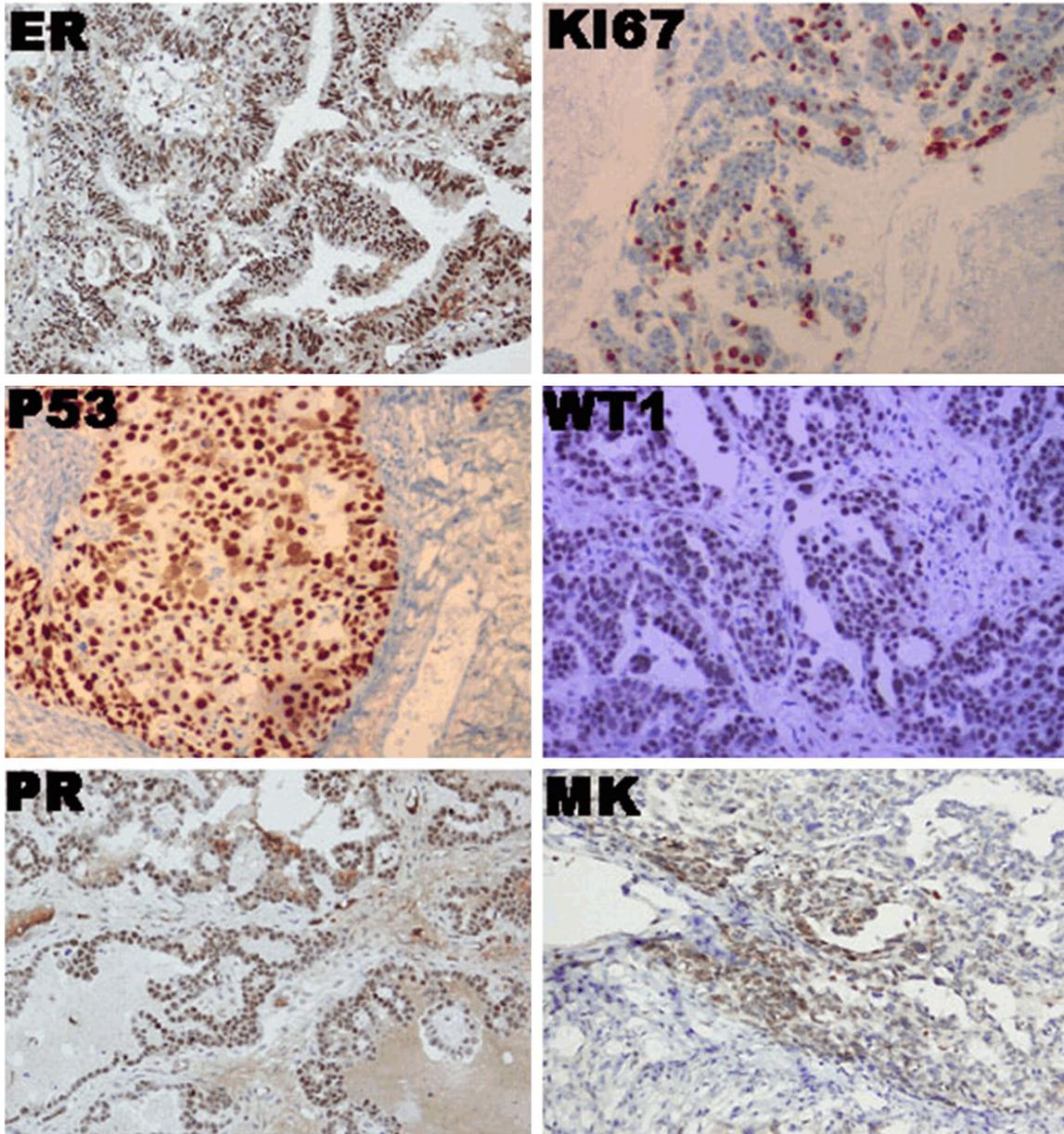
Characteristics	FIGO stage		Metastasis	
	I/II	III/IV	Yes	No
Age (years)				
Mean	56.35	54.58	54.91	56.07
Range	37–70	42–80	42–79	42–80
Age distribution				
≤50	13 (10.9%)	29 (24.4%)	30 (25.2%)	12 (10.1%)
>50	38 (31.9%)	39 (32.8%)	45 (37.8%)	32 (26.9%)
Histology				
Serous	28 (23.5%)	47 (39.5%)	47 (39.5%)	28 (23.5%)
Others	23 (19.3%)	21 (17.7%)	28 (23.6%)	16 (13.5%)
Ascites				
≤300 mL	40 (33.6%)	41 (34.5%)	48 (40.3%)	33 (27.7%)
>300 ml	11 (9.2%)	27 (22.7%)	27 (22.7%)	11 (9.2%)
Tumor grade				
G1–G2	17 (14.3%)	20 (16.8%)	18 (15.1%)	19 (16%)
G3	34 (28.6%)	48 (40.3%)	57 (47.9%)	25 (21%)

Legend: FIGO, The International Federation of Gynecology and Obstetrics.

**Table S2.** Contingency analysis of the levels of serum markers in relation to known prognostic factors in patients with ovarian cancer

Characteristics	Markers				
	CA-125 (U/mL)	CA19-9 (U/mL)	CA15-3 (U/mL)	AFP (ng/mL)	CEA (ng/mL)
Age distribution					
≤50	479.03	36.36	60.84	2.49	5.23
>50	332.71	42.32	36.44	3.72	4.66
<i>P</i>	0.28	0.73	0.76	0.13	0.84
Histology					
Serous	554.57	35.52	46.46	3.54	1.46
Others	174.52	40.21	21.64	3.02	5.77
<i>P</i>	0.04	0.85	0.02	0.47	0.00
Ascites					
≤300 mL	452.77	34.07	29.39	3.20	3.05
>300 mL	576.09	50.36	99.90	2.94	5.54
<i>P</i>	0.56	0.56	0.00	0.59	0.38
FIGO					
I/II	255.67	57.83	34.07	3.34	4.76
III/IV	530.69	24.30	53.15	3.49	5.04
<i>P</i>	0.05	0.08	0.13	0.78	0.92
Grade					
G1–G2	434.61	8.78	22.38	4.27	4.59
G3	522.26	24.36	53.45	1.95	1.45
<i>P</i>	0.70	0.62	0.05	0.01	0.09
Metastasis					
Yes	517.25	74.70	52.72	3.12	4.65
No	237.51	40.73	34.93	3.55	5.15
<i>P</i>	0.03	0.37	0.17	0.38	0.80

Legend: AFP, alpha-fetoprotein; CA, cancer antigen; CEA, carcinoembryonic antigen.



**Figure S1.** Representative immunohistochemical examples of positive staining for various tumor markers in epithelial ovarian cancer (EOC) tissues. The pictures were taken using an Olympus BX50 microscope (magnification  $\times 100$ ). Legend: ER, estrogen receptor; KI67, antigen KI-67; p53, tumor protein 53; WT1, Wilms tumor protein; PR, progesterone receptor; MK, midkine.