

Original Article

High expression of S100P is associated with unfavorable prognosis and tumor progression in patients with epithelial ovarian cancer

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Abstract: Accumulating evidence has demonstrated that S100P is involved in the tumorigenesis and progression of multiple cancers. In the current study, we evaluated the expression of S100P in epithelial ovarian cancer and assessed its relevance to clinicopathological characteristics. Moreover, we investigated the biological effects of S100P using A2780 and SKOV3 cells. S100P expression was significantly increased in epithelial ovarian cancer specimens compared with fallopian tube tissues and normal ovary tissues. And high expression of S100P in epithelial ovarian cancer samples was significantly associated with tumor stage ($P<0.001$), serum CA125 level ($P=0.026$), residual tumor ($P<0.001$), ascites ($P<0.001$) and lymph nodes metastasis ($P<0.001$). Multivariate Cox analysis showed that S100P expression was an independent prognostic factor of overall survival (OS) and progression free survival (PFS) ($P=0.017$ and 0.031 , respectively). Functional assays showed that overexpression of S100P promoted cell proliferation and cell cycle progression but did not affect cell migration and invasion in A2780 and SKOV3 cells. These data suggest that S100P may contribute to tumor development in epithelial ovarian cancer and could be a useful marker for the prognosis of epithelial ovarian cancer patients.

Keywords: Ovarian cancer, S100P, survival, prognosis, cell proliferation

Introduction

Epithelial ovarian cancer (EOC) is the most common form of ovarian cancer. It is the leading cause of cancer death involving the female genital tract [1]. The overall survival of women with ovarian cancer has not changed in more than 50 years [2]. The high mortality of EOC is mainly due to late-stage diagnosis for more than 70% of patients. These patients have missed the optimal timing for surgery and a five-year survival rate was below 40% [3]. Similar to other human malignancies, the tumorigenesis and tumor progression of EOC are caused by numerous reproductive, environmental, and genetic risk factors [4]. The origin and pathogenesis of EOC have been investigated but still poorly understood. An improved understanding of the molecular mechanisms

underlying ovarian cancer may lead to the development of more effective therapeutic strategies.

S100 is a multigenic family of EF-hand calcium-binding proteins. The altered expression of S100 proteins was associated with tumorigenesis and tumor progression. Recently, there is growing interest in the S100 protein family and their relationship with different cancers [5]. S100P is a member of the S100 family, which functions through both extracellular and intracellular mechanisms and participates in various human pathologies including cancer. And S100P was found to be over expressed in many different cancers and is considered a potential target for cancer therapy. Increased levels of S100P expression have been found to correlate with poor survival in breast cancer, lung cancer,

Table 1. The clinicopathologic characteristics of patients

Clinical Variable	NO. of patients (%)
All cases	221
Age (years), median (range)	52 (32-77)
Histological type	
Serous	158 (71.49%)
Mucinous	26 (11.77%)
Endometrioid	20 (9.05%)
Clear cell	17 (7.69%)
FIGO stage	
I	34 (15.38%)
II	47 (21.27%)
III	118 (53.39%)
IV	22 (9.95%)
Tumor grade	
G1	18 (8.14%)
G2	46 (20.81%)
G3	157 (71.04%)
Residual tumor size (cm)	
<2 cm	171 (77.38%)
≥2 cm	50 (22.62%)
Lymph node metastasis	
Negative	136 (61.54%)
Positive	85 (38.46%)
Two-tier system	
Type I	96 (43.44%)
Type II	125 (56.56%)
Serum CA125 level (U/ml)	
<35	54 (24.43%)
≥35	167 (75.57%)
Chemotherapy resistance ^a	
Present	43 (20.00%)
Absent	172 (80.00%)
Ascites (ml)	
<500	108 (48.87%)
≥500	113 (51.13%)

^asix patients with stage I and Grade I didn't undergo chemotherapy.

pancreatic cancer, hepatocellular carcinoma, gastric cancer and cholangio carcinoma [6-11]. A number of studies have strongly linked S100P to cell proliferation, invasion, migration and metastasis in cancers [6, 12-15]. And S100P exerts its functions by binding to and activating the Receptor for Advance Glycation End-products (RAGE).

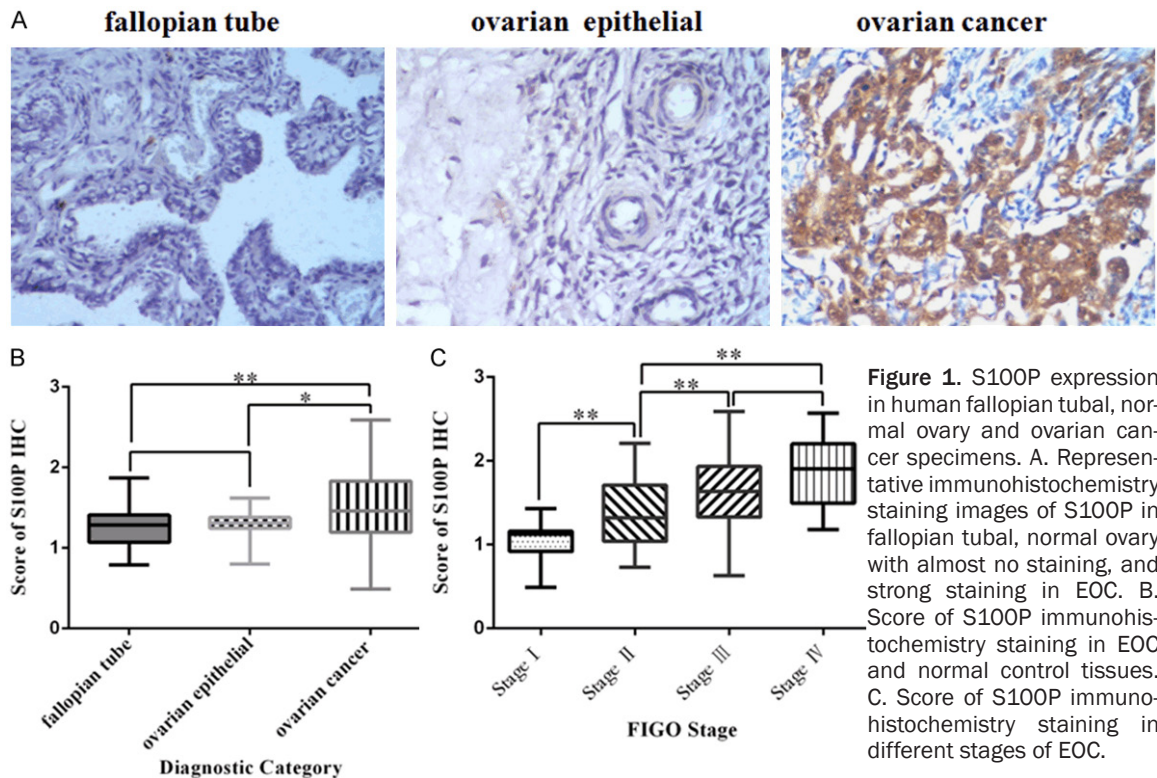
To date, however, the status of S100P expression and its clinical prognostic relevance in EOC

have not been fully elucidated. In this study, we detected the expression of S100P protein in ovarian cancer with tissue microarray (TMA) by immunohistochemistry (IHC) and evaluated the correlation of S100P expression with the clinicopathologic features of EOC. Next, we studied its biological function in ovarian cancer cell lines by lentiviral-mediated overexpression of S100P in A2780 and SKOV3 cells.

Materials and methods

Patient samples

221 Formalin-fixed paraffin-embedded (FFPE) blocks from EOC patients were collected from November 2007 to August 2013. Patients were enrolled in the Affiliated Hospital of Qingdao University. In addition, 30 normal ovarian tissue blocks and 32 fallopian tubal tissue blocks obtained from exeresis (surgical removal) from non-ovary diseases during the same time period were used as controls. The cancer cases selected were based on the availability of resected tissue and follow-up data. These patients aged from 43 to 58 years, with a median age of 52 years. The pathological diagnoses of all epithelial ovarian cancer patients were confirmed by two veteran pathologists respectively. All patients had no history of radiotherapy and immunotherapy. We categorized these tissue samples according to several clinical and pathological parameters: age at first diagnosis, FIGO stage, histological subtype, histological grade, volume of ascites, serum CA125 level, residual tumor mass after surgery and responses to platinum-based chemotherapy (**Table 1**). This study was approved by ethics committee of the Affiliated Hospital of Qingdao University. All patients underwent cytoreductive surgery with maximal surgical effort and adjuvant platinum-based chemotherapy except six patients with stage I and Grade I. All patients were surgically staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging [16]. Progression of the disease was diagnosed on the basis of the value of CA125 and CT results according to the RECIST criteria [16, 17]. The documentation of residual tumor mass was also assessed prospectively at the end of the surgery with the main surgeon. The OS was defined as the interval from the end of treatment to death due to any cause or to the date of last contact. PFS was defined as the period after the conclusion



of treatment to proven local recurrence or distant metastasis.

The patients' sensitivity to platinum analogues was evaluated according to the following criteria: 1. The platinum sensitive patients, the disease-free time was longer than 6 months after first-line chemotherapy. 2. The platinum resistant patients, the disease-free time was shorter than 6 months after first-line chemotherapy. 3. The platinum refractory patients, disease progression occurred during the first-line chemotherapy [18].

A new two-tier system was used to categorize various types of epithelial ovarian cancers into two groups. Two groups were designated Type I and Type II. Type I tumors comprise low-grade serous, low-grade endometrioid, mucinous, and clear cell carcinomas. Type II tumors include high-grade serous, high-grade endometrioid, undifferentiated carcinomas, and malignant mixed mesodermal tumors (carcinosarcomas) [2].

Immunohistochemistry staining

Tissue sections were dewaxed with xylene and rehydrated through gradient ethanol into water.

After endogenous peroxidase activity was quenched with 3% H_2O_2 for 30 minutes, heated in citrate buffer (pH 6.0) at 95°C for 25 min, and cooled at room temperature. After washing with PBS, nonspecific antibody binding was blocked by preincubating slides in 10% normal goat nonimmune serum at 37°C for 30 minutes. Sections were incubated with the polyclonal primary antibody against S100P (11803-1-AP, Proteintech) at 1:100 dilution overnight at 4°C. IHC was performed using the PV-9000 Polymer Detection System for Immunohistological Staining kit (Beijing Golden Bridge Biotechnology Company). DAB was used to visualize the reaction, followed by counterstaining with Hematoxylin. All slides were scanned by Aperio scanning system (Aperio, San Diego, USA) and the Aperio Image Scope software was employed for quantitative analysis of S100P protein expression. About 4 to 6 different parts of the slide were randomly selected for analysis.

Cell lines and cell culture

The human EOC cell lines 3AO, A2780, SKOV3, and ES-2 were all purchased from the American Type Culture Collection (ATCC; Manassas, VA). A2780 and ES-2 cells were maintained in

Table 2. Correlation between S100P expression and clinicopathological characteristics in EOC patients

Clinical Variable	No. of patients	S100P expression Median (range)	P ^b
All cases	221		
Age at Diagnosis (years)			0.173
<52	108	1.48 (0.71-2.57)	
≥52	113	1.45 (0.49-2.59)	
Histological type			0.380
Serous	158	1.475 (0.49-2.42)	
Mucinous	26	1.435 (0.73-2.57)	
Endometrioid	20	1.36 (0.61-2.11)	
Clear cell	17	1.49 (0.93-2.59)	
FIGO stage			<0.001
I	34	1.125 (0.49-1.43)	
II	47	1.32 (0.73-2.21)	
III	118	1.635 (0.63-2.59)	
IV	22	1.905 (1.18-2.57)	
Tumor grade			0.389
G1	18	1.38 (0.78-2.59)	
G2	46	1.365 (0.63-2.42)	
G3	157	1.47 (0.49-2.57)	
Residual tumor size (cm)			<0.001
<2 cm	171	1.41 (0.49-2.42)	
≥2 cm	50	1.745 (0.74-2.59)	
Lymph node metastasis			<0.001
Negative	136	1.32 (0.49-2.42)	
Positive	85	1.67 (0.63-2.59)	
Two-tier system			0.219
Type I	96	1.415 (0.63-2.59)	
Type II	125	1.47 (0.49-2.42)	
Serum CA125 level (U/ml)			0.026
<35	54	1.41 (0.49-2.59)	
≥35	167	1.47 (0.63-2.57)	
Chemotherapy resistance ^a			0.099
Present	43	1.59 (0.74-2.59)	
Absent	172	1.46 (0.49-2.42)	
Ascites (ml)			<0.001
<500	108	1.315 (0.49-2.59)	
≥500	113	1.65 (0.63-2.57)	

^aSix patients with stage I and Grade I didn't undergo chemotherapy.

^bMann-Whitney *U* and Kruskal-Wallis nonparametric test were used for comparing different groups.

and 100 U/ml penicillin. All cell lines were cultured in complete medium in a 5% CO₂ 37°C incubator. The medium was changed at alternate days and cells were split before they reached confluency.

Lentiviral construct, production of lentiviruses and lentiviral transduction

Lentiviral vector carrying full-length cDNA of human S100P (Ex-I0533-LV101) and control vector (Ex-EGFP-LV101) were purchased from Gene-copoeia. 10 cm plate containing 6×10⁶ 293T cells was transfected using FuGENE HD (Roche) with 5 µg control or S100P lentiviral vector, 3.75 µg pCMV Δ8.91 and 1.25 µg VSVG. Supernatants were collected at 48 hours after transfection and frozen at -70°C. For lentiviral transduction, 2×10⁵ cells/well were seeded in 6-well culture plates and infected the following day with lentiviruses. To generate stable cell lines, cells were selected for two weeks with 500 µg/ml Geneticin.

Quantitative real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction was performed using Power SYBRs Green PCR Master Mix (Applied Biosystems) on the StepOne Plus™ Real-Time PCR System (Applied Biosystems) following the manufacturer's instructions. Real-Time PCR was done using the following primers: S100P, F: 5'-GAAGGAGCTACCAGGCTTCC-3' and R: 5'-CTCACTGAAGTCCACCTGGG-3'. β-actin, F: 5'-GGCGGCACCA-CATGTACCCT-3' and R: 5'-AGGGGCC-GGACTCGTCATACT-3'.

Western blotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with the appropriate primary antibodies at 4°C overnight. After washing, the membranes were incubated with secondary

Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 U/ml streptomycin, and 100 U/ml penicillin. 3AO and SKOV3 cells were cultured in RPMI 1640 medium with 10% FBS, 100 U/ml streptomycin,

Table 3. Univariate and multivariate analyses of factors affecting PFS in patients with EOC

Outcome Variables	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P
Age at diagnosis (years)				
<52	0.856 (0.623-1.176)	0.39		
≥52				
Two-tier system				
Type I	1.158 (0.84-1.597)	0.372		
Type II				
FIGO stage				
I-II	2.359 (1.654-3.365)	<0.001	1.816 (1.149-2.870)	0.011
III-IV				
Tumor grade				
G1+G2	1.554 (1.073-2.252)	0.02	1.421 (0.975-2.070)	0.068
G3				
Residual tumor size (cm)				
<2 cm	2.833 (1.993-4.027)	<0.001	1.604 (1.037-2.481)	0.034
≥2 cm				
Lymph node metastasis				
Negative	1.68 (1.215-2.324)	0.002	0.639 (0.417-0.979)	0.040
Positive				
Ascites (ml)				
<500	1.351 (0.98-1.863)	0.067		
≥500				
Serum CA125 level (U/ml)				
<35	1.60 (1.081-2.367)	0.019	1.192 (0.796-1.786)	0.394
≥35				
Chemotherapy resistance				
Present	0.3 (0.001-1.9)	<0.001	5.5 (0.001-9.7)	0.749
Absent				
S100P				
<1.46 ^a	2.095 (1.502-2.921)	<0.001	1.497 (1.037-2.161)	0.031
≥1.46				

^aMedian of relative expression. HR, hazard ratios; CI, confidence intervals.

antibody at a dilution of 1:5,000 for 1 hour at room temperature. Proteins were detected with the enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). Anti-S100P antibody (11803-1-AP, Proteintech) was used. β -actin antibody (A5316, Sigma) was used to test for equal loading.

Fluorescence-activated cell sorting analysis

Cells were washed in PBS and fixed in methanol overnight. Subsequently, cells were washed and resuspended in PBS containing 50 μ g/mL propidium iodide, 100 μ g/mL RNase, and 0.1%

Nonidet P-40 for 30 minutes at 37°C. The distribution of cells in different phases of the cell cycle was determined by measuring the nuclear DNA content using a FACS Calibur cell flow cytometer (Becton, Dickinson and Company).

Cell proliferation assay

MTS assay was applied to assess cell proliferation as instructed by the manufacturer (Promega). Cells were seeded at 3×10^3 cells/well in 100 μ l/well using 96-well culture plates. The absorbance of the samples was measured at 490 nm on a scanning multi-well spectrophotometer. The experiment was repeated 3 times. Cell proliferation was compared at four time point (6 h, 24 h, 48 h, and 72 h) after the cells were seeded.

Cell migration and invasion assay

In vitro cell migration and invasion assays were performed as described previously [19]. Cells growing in the log phase were trypsinized, re-suspended in serum-free medium, and seeded into Boyden chambers (8 μ m pore size with polycarbonate membrane). The chambers were then inserted into transwell apparatus (Costar, Cambridge, MA, USA). The chambers were

coated with Matrigel (BD Biosciences, San Jose, USA) when cell invasion assay was done. Medium with 10% FBS (600 μ l) was added to the lower chamber. After incubation of 48 hours, cells on the top surface of the insert were removed by wiping with cotton swab. Cells that migrated to the bottom surface of the insert were stained in 0.3% crystal violet for 30 min, rinsed in PBS and then subjected to microscopic inspection. Images of four random fields (10 \times) were captured from each membrane, and the number of migratory or invasive cells was counted. The migration and invasion results were normalized by cell proliferation under the

Table 4. Univariate and multivariate analyses of factors affecting OS in patients with EOC

Outcome Variables	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P
Age at diagnosis (years)				
<52	0.891 (0.632-1.255)	0.508		
≥52				
Two-tier system				
Type I	1.158 (0.84-1.597)	0.414		
Type II				
FIGO stage				
I-II	3.37 (2.26-5.026)	<0.001	1.831 (1.069-3.137)	0.028
III-IV				
Tumor grade				
G1+G2	1.512 (1.014-2.256)	0.043	1.191 (0.782-1.812)	0.416
G3				
Residual tumor size (cm)				
<2 cm	2.369 (1.635-3.433)	<0.001	0.919 (0.596-1.417)	0.702
≥2 cm				
Lymph node metastasis				
Negative	2.407 (1.68-3.448)	<0.001	1.255 (0.789-1.994)	0.337
Positive				
Ascites (ml)				
<500	1.988 (1.394-2.836)	<0.001		
≥500				
Serum CA125 level (U/ml)				
<35	1.74 (1.143-2.650)	0.01	1.343 (0.853-2.114)	0.203
≥35				
Chemotherapy resistance				
Present	7.315 (4.902-10.917)	<0.001	7.792 (4.92-12.34)	<0.001
Absent				
S100P				
<1.46 ^a	3.325 (2.252-4.090)	<0.001	1.763 (1.106-2.811)	0.017
≥1.46				

^aMedian of relative expression. HR, hazard ratios; CI, confidence intervals.

same treatment conditions. Triplicate assays were used or each experiment.

Statistical analysis

Statistical analyses were performed with the SPSS software version 22.0 (SPSS Inc., Chicago, IL). Normality of distribution was assessed using the Shapiro-Wilk test. Because the distribution was not normal, comparisons between S100P levels in EOC and normal tissues and clinicopathologic variables were made by Kruskal-Wallis test or Mann-Whitney *U* test where appropriate. Survival curves were

generated using Kaplan-Meier estimates. In the univariate analysis, each parameter was categorized for subsequent statistical analysis. Only variables with a *P* value <0.05 in the univariate analysis were included in the multivariate model. Hazard ratios (HR) and 95% confidence intervals (CI) computed from multivariate Cox regression models were used to investigate the relationships between clinico-pathologic characteristics and survival. A two-tailed *P* value <0.05 was considered statistically significant.

Results

S100P is overexpressed in EOC tissues compared with normal control and associated with tumor stage and lymph node metastasis

EOC is a very aggressive gynecological tumor. In recent years, with the full realization of the genesis for ovarian cancer, it is strongly suggested that high grade ovarian cancer originates not from the surface of the ovary, but from the epithelial layer of the neighboring fallopian tube epithelium [20, 21]. Therefore, according to the MDACC two-tier grading system, fallopian tube

tissues were taken for control group together with normal ovaries. In this study, S100P expression was examined by IHC on sections isolated from 30 normal ovaries, 32 fallopian tubal tissues, and 221 EOCs. All normal ovary tissues and fallopian tubal tissues exhibited absent or weak S100P expression, whereas the majority of EOC tissues showed positive S100P expression. And S100P immunostaining was observed in the cell membrane and cytoplasm of EOCs (**Figure 1A**). EOC tissues had higher S100P expression levels than in normal ovary tissues and fallopian tubal tissues (*P*=0.023 and 0.003, respectively), but it was

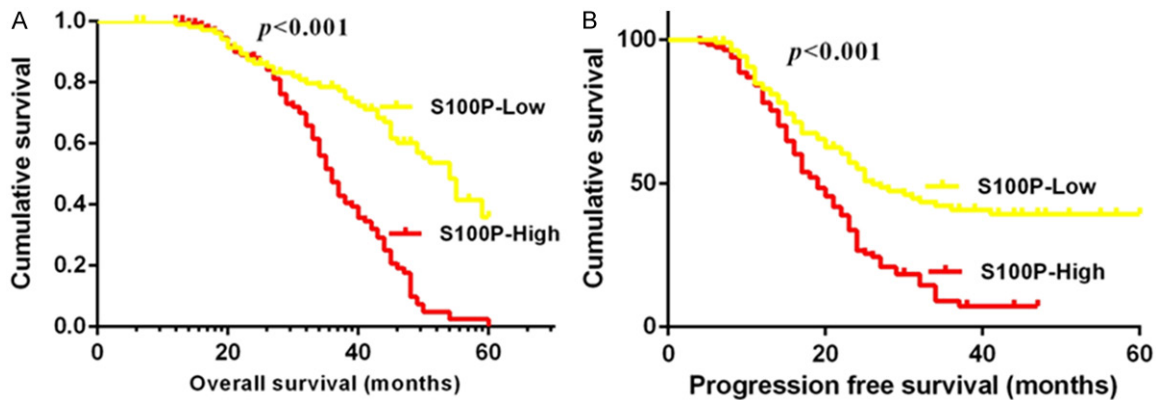


Figure 2. Elevated S100P expression is associated with shorter overall survival and progression free survival. A. Overall survival curve of S100P protein expression in tumor tissues of EOC patients (Kaplan-Meier analysis, $P < 0.001$). B. Progression free survival curve of S100P protein expression in tumor tissues of EOC patients (Kaplan-Meier analysis, $P < 0.001$).

not different between normal ovary tissues and fallopian tubal tissues ($P > 0.05$) (**Figure 1B**). To further determine the clinical significance of the elevated S100P protein expression in EOC tissues, we analyzed the correlation between S100P expression and the clinicopathological parameters of EOC patients. There was a significant difference between stage I and stage II, and between stage II and stage III ($P = 0.003$ and 0.003 , respectively). However, there was no difference in S100P expression level between stage III and stage IV. And S100P exhibited higher expression in EOC tissues with advanced stage (III~IV) than those with early stage (I~II) ($P < 0.001$, **Figure 1C**). Furthermore, high S100P expression in EOC tissues was also significantly associated with lymph node metastasis ($P < 0.001$), preoperative serum CA125 level ($P = 0.026$), residual tumor ($P < 0.001$), and ascites ($P < 0.001$). In contrast, S100P expression in EOC tissues was not correlated with age at diagnosis, two-tier system, chemotherapy resistance, histological type, and grade ($P > 0.05$) (**Table 2**).

S100P, a potential novel prognostic marker in EOC

The median follow-up interval was 36 months. Fourteen patients were lost during follow-up. In univariate survival analyses for OS and PFS, 221 EOC patients were divided into two groups based on median of S100P expression score in tumors, representing low (scores < 1.46) and high (scores ≥ 1.46) expression of S100P. The median OS for the high S100P expression

group (114 patients, 83 events) was 33 month; however, the low S100P expression group had significantly longer survival (42 months) (107 patients, 49 events). The median PFS was 18.5 month for the high S100P expression group but 24 month for the low S100P expression group.

The prognostic values of age at diagnosis, histological type, grade, FIGO stage, residual tumor, lymph node metastasis, ascites, preoperative serum CA125 level, two-tier system, and S100P expression were further tested in univariate and multivariate analyses for both PFS and OS (**Tables 3, 4**). Kaplan-Meier survival curves indicated that patients with high S100P expression were more likely to have a shorter PFS and OS compared with patients with low S100P expression ($P < 0.001$ and $P < 0.001$) (**Figure 2A, 2B**). FIGO stage, grade, residual tumor size, lymph node metastasis, preoperative CA125 level, and chemotherapy resistance were also associated with a higher risk of disease recurrence in the univariate analysis ($P < 0.001$, $P = 0.02$, $P < 0.001$, $P = 0.002$, $P = 0.019$, and $P < 0.001$, respectively). And FIGO stage, grade, residual tumor size, lymph node metastasis, preoperative CA125 level, ascites and chemotherapy resistance were also associated with OS in the univariate analysis ($P < 0.001$, $P = 0.043$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P = 0.01$, and $P < 0.001$, respectively). In the multivariate analysis of PFS, high S100P immunoreactivity exhibited an independent, negative prognostic function in the full series of patients ($P = 0.031$). In the multivariate analysis of OS, high S100P immunoreactivity was inde-

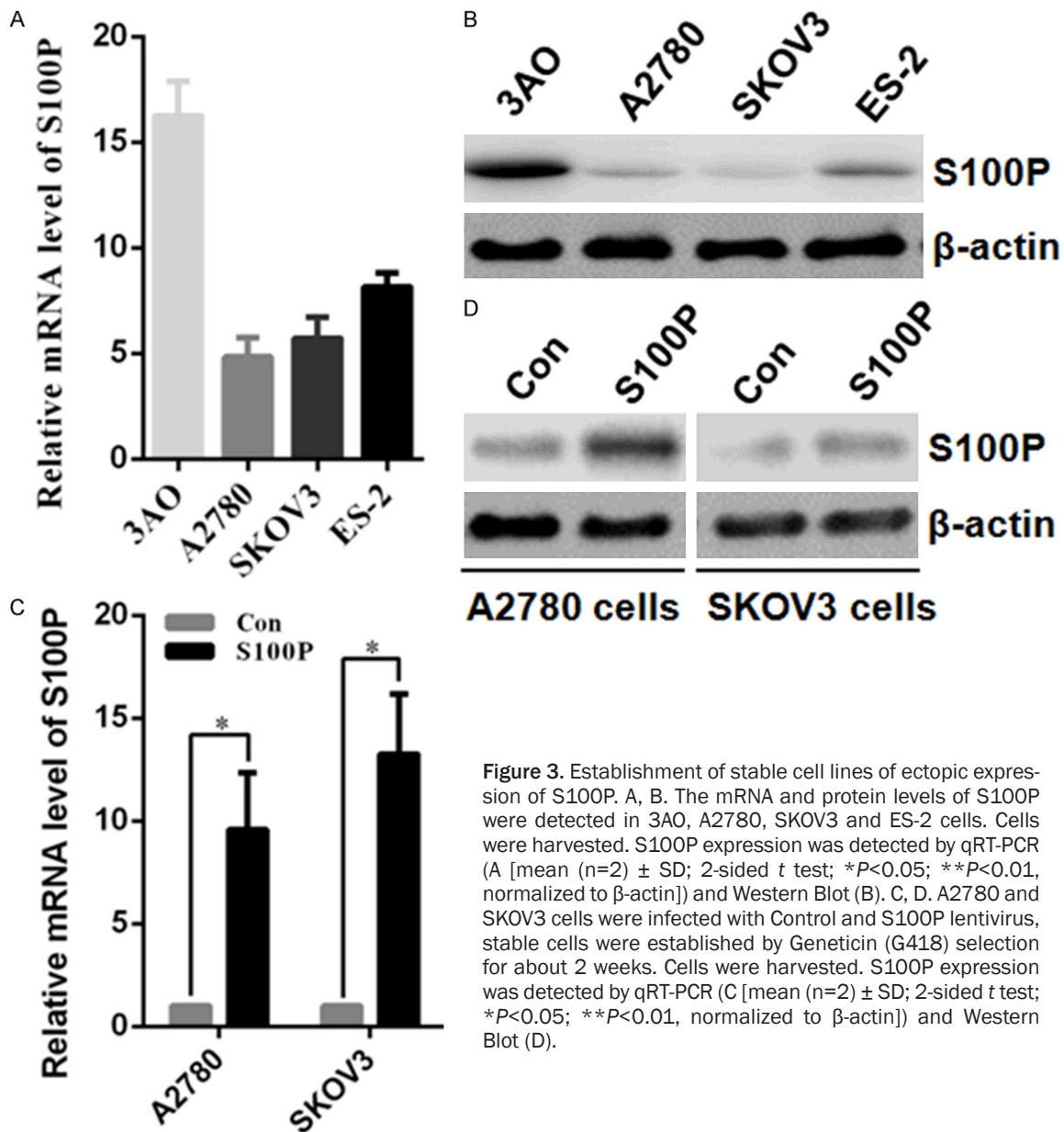


Figure 3. Establishment of stable cell lines of ectopic expression of S100P. A, B. The mRNA and protein levels of S100P were detected in 3AO, A2780, SKOV3 and ES-2 cells. Cells were harvested. S100P expression was detected by qRT-PCR (A [mean (n=2) ± SD; 2-sided t test; * $P < 0.05$; ** $P < 0.01$, normalized to β-actin]) and Western Blot (B). C, D. A2780 and SKOV3 cells were infected with Control and S100P lentivirus, stable cells were established by Geneticin (G418) selection for about 2 weeks. Cells were harvested. S100P expression was detected by qRT-PCR (C [mean (n=2) ± SD; 2-sided t test; * $P < 0.05$; ** $P < 0.01$, normalized to β-actin]) and Western Blot (D).

pendently correlated with an unfavorable prognosis with respect to OS ($P = 0.017$). FIGO stage, residual tumor, and lymph node metastasis were also found to be associated with a higher risk of disease recurrence in all patients ($P = 0.011$, $P = 0.034$ and $P = 0.04$, respectively). Meanwhile, FIGO stage and chemotherapy resistance were also unfavorable prognostic indicators with respect to OS ($P = 0.028$ and $P < 0.001$, respectively).

S100P promotes G2/M phase progression and cell growth in EOC cells

The above histological studies showed that S100P may play an important role in the tumor-

igenesis and progression of EOC. So we set out to investigate the potential role of S100P in the development of a malignant phenotype in EOC cells by altering intracellular S100P expression. Firstly, we detected S100P mRNA and protein expression in four EOC cell lines including 3AO, A2780, SKOV3 and ES-2. We found a high level of S100P expression in 3AO cells but a low level in A2780 and SKOV3 cells (Figure 3A and 3B). To characterize the role of S100P in the development of malignant phenotype, we over expressed S100P using lentiviral-mediated gene delivery in A2780 and SKOV3 cells. Over expression of S100P in A2780 and SKOV3 cells were confirmed at the mRNA and protein levels

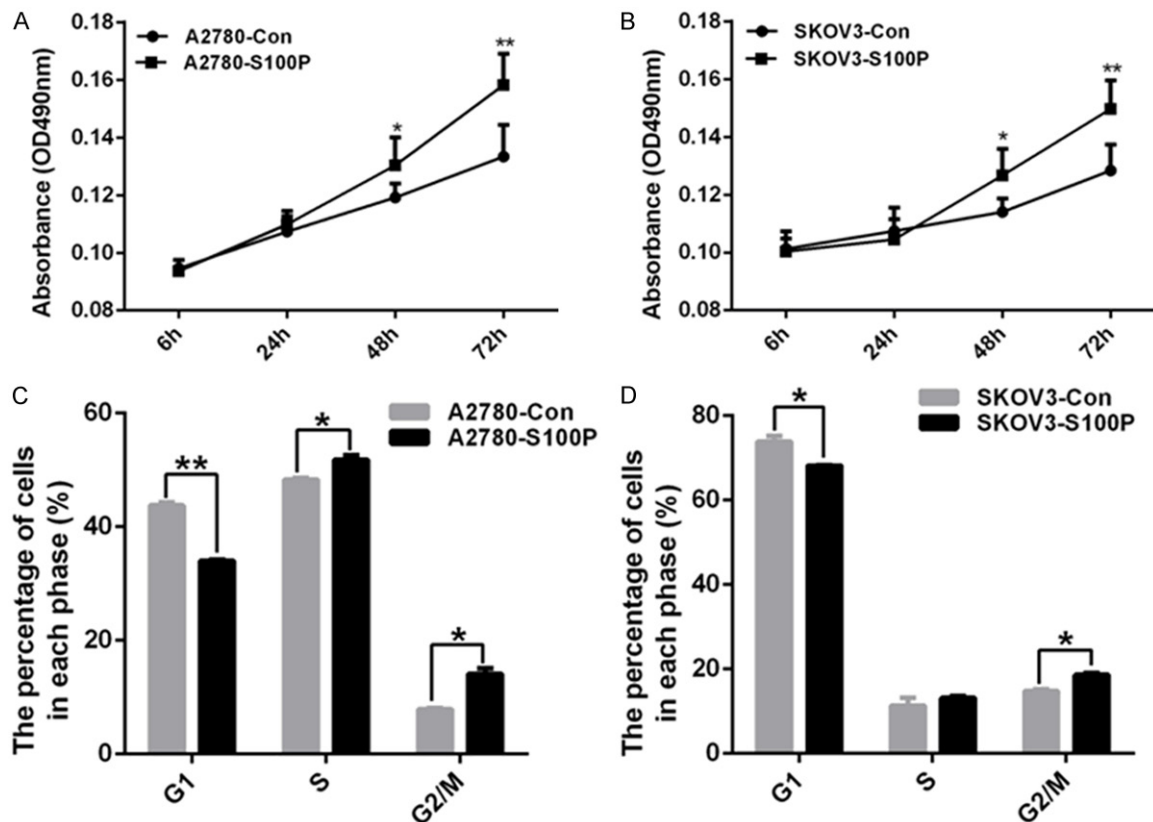


Figure 4. S100P promotes ovarian cancer cell proliferation and cell cycle progression. A, B. Cell proliferation was determined by MTS assay. And overexpression of S100P markedly promoted cell proliferation of A2780 and SKOV3 cells. C, D. Cell-cycle distribution was analyzed by FACS, and a significant G2/M phase increase and G1 phase decrease were observed in S100P-overexpressed cells compared with their corresponding control group [mean (n=2) \pm SD; 2-sided *t* test; **P*<0.05; ***P*<0.01]. C. A2780 cells; D. SKOV3 cells.

by qRT-PCR and Western Blot analysis (Figure 3C, 3D). Previous studies have documented a role for S100P in regulation of cell cycle progression and cell growth [22, 23]. To determine the effect of S100P on cell proliferation, MTS assay was performed on A2780 and SKOV3 cells. The results showed that cell proliferation was significantly enhanced in S100P-overexpressing cells versus the corresponding control cells (Figure 4A, 4B). To verify whether the action of S100P on the proliferation of EOC cells is associated with a change in cell cycle distribution, cell cycle analysis was performed using flow cytometry. The results showed that G2/M phase cells increased with a parallel decrease in G1-phase cells in S100P-overexpressed A2780 and SKOV3 cells (Figure 4C, 4D). These results fully demonstrated that S100P was involved in the regulation of cell cycle progression and cell proliferation. Since previous literatures have also demonstrated that S100P is involved in the regulation of cell

migration and invasion [12, 24, 25]. We detect the effect of S100P over expression on the capability of migration and invasion in A2780 and SKOV3 cells. However, over expression of S100P did not have impact on the migratory and invasive capacity (Figure 5A-F).

Discussion

S100P protein is an example of calcium-modulated protein that intervenes in the fine tuning of a relatively large number of specific intracellular and extracellular activities. S100P has received increasing attention and accumulating evidence has suggested its significant role during the development and progression of different cancers [10, 26-31]. Moreover, the potential roles of S100P as diagnostic marker, prognostic indicator, and therapy target have been suggested in multiple cancers [32]. However, its role in human EOC has not yet been determined. Here, we measured and com-

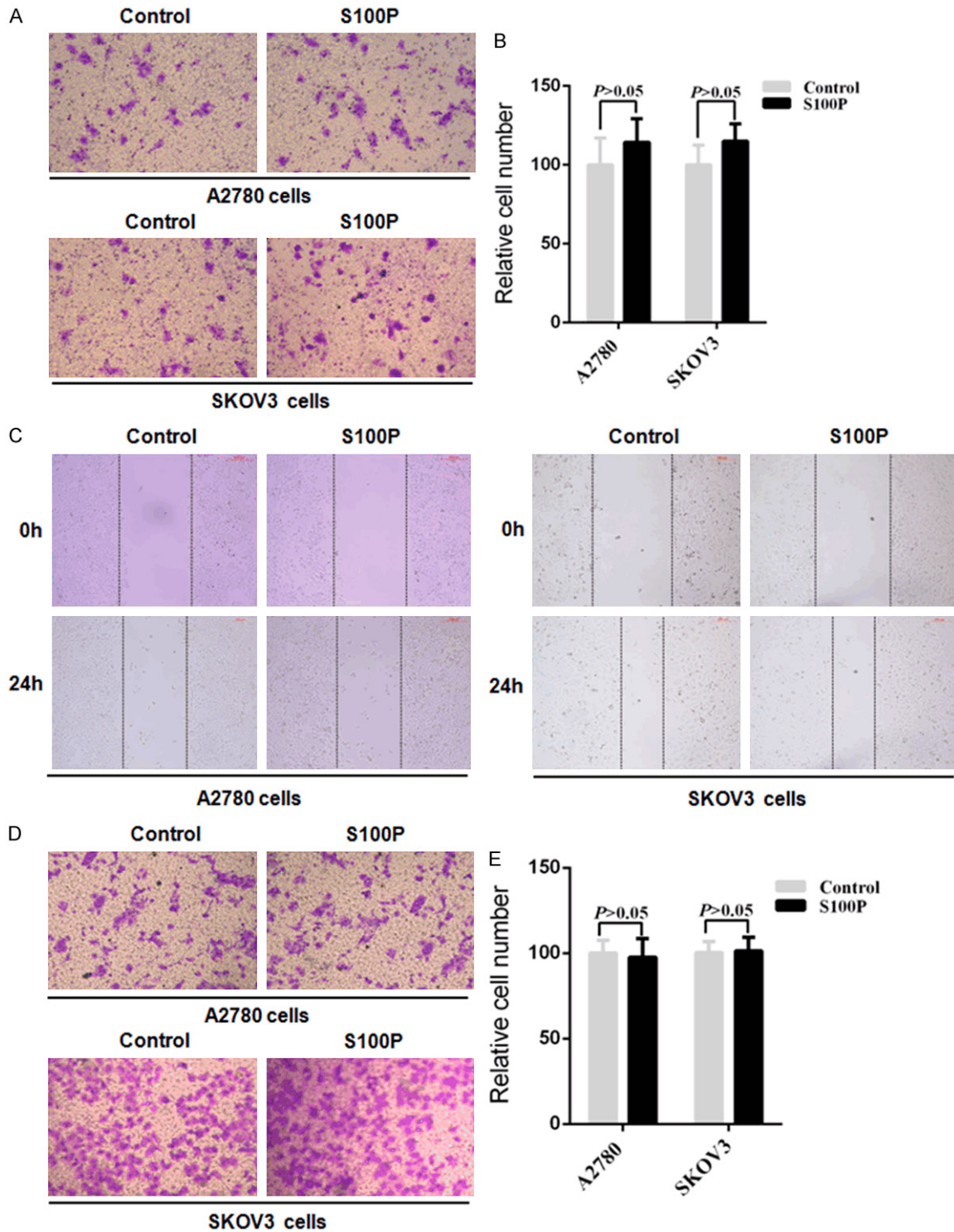


Figure 5. S100P has no impact on ovarian cancer cell migratory and invasive capabilities. A. Representative images of cell migration using transwell assay (Upper, A2780 cells, Below, SKOV3 cells). B. Quantitative results of cell migration using transwell assay. The stained cells were manually counted from 4 randomly selected fields and normalized with cell proliferation. C. The effect of S100P on cell migration was examined in wound-healing assay. Confluent cells were scratched with a 10 μ l pipette tip and photographed at time zero and 24 h (Left Panel, A2780 cells, Right Panel, SKOV3 cells). D. Representative images of cell invasion (Upper, A2780 cells, Below, SKOV3 cells). E. Quantitative results of cell invasion assays.

pared the expression levels of S100P in human EOC tissues by immunohistochemistry. Our results showed a significantly higher expression of S100P in EOC than that in fallopian tube tissues and normal ovary tissues. Expression of S100P was weak in control groups, but increased in early-stage tumors, and reached the highest level in advanced stage tumors. Statistical analysis revealed that the high expression of S100P was positively correlated with advanced tumor FIGO stage and positive lymph nodal involvement, suggesting a crucial role of S100P in the progression of EOC. Since serum cancer antigen 125 (CA125) has become well established as a biomarker for EOC and has shown to have an important role in initial diagnosis, during therapy as a surrogate of clinical response and during follow-up. And many patients with advanced ovarian cancer have ascites. The correlation between S100P expression with CA125 levels and ascites was analyzed. The results showed that S100P expression was associated with high CA125 and ascites levels. These results provide preliminary evidence that elevated S100P expression may indicate a high risk for EOC development and progression.

To further explore the potential prognostic value of S100P in EOCs, the correlation between S100P expression and PFS and OS in EOC patients was analyzed. The results demonstrated a significant correlation between high S100P immunoreactivity and shorter overall and disease-free survival, suggesting S100P may serve as an important prognostic marker and an attractive therapeutic target in ovarian cancer.

Failure of EOC treatment is largely due to chemotherapy resistance, an important prognostic index for EOC patients in clinic [33]. Response to first line chemotherapy was defined according to the last time patients received platinum-based compounds. In the study, all patients except 6 patients with FIGO I and Grade I were treated with adjuvant paclitaxel-based chemotherapy after cytoreductive surgery. They received 6 cycles of this combined regimen. To date, many studies have demonstrated that altered expression of S100P is associated with the drug-resistant of various human cancers. For example, ectopic expression of S100P increases drug resistance in gastric cancer and decreases chemosensitivity to 5-FU *in vitro* in

colorectal cancer cells [10, 25]. In contrast, knockdown of S100P decreased sensitivity to cisplatin [34]. On the contrary, over expression of S100P in resistant bladder cells resulted in an increased sensitivity to cisplatin [35]. Over expression of S100P sensitized OVCAR3 cells for chemotherapeutic drugs and knockdown of S100P leads to increased drug resistance in OVCAR3 cells [36, 37]. The disparity among these studies may result from different functional roles of S100P in a tissue/cell dependent manner. In our study, we analyzed the correlation between S100P expression with chemosensitivity in ovarian cancer samples and the results showed that there was no significant difference of S100P expression between the chemotherapy-resistant group and the chemotherapy-sensitive group. In future studies, we will further examine the effect on chemotherapeutic drugs using *in vitro* experimental system.

Previous studies have demonstrated that S100P plays an important role in facilitating tumor cell proliferation. Silencing of S100P suppressed cell proliferation and augmented cell apoptosis in Hep3B cells [22]. And S100P knockdown decreased the S-phase fraction of cisplatin sensitive cell lines and suppressed cell proliferation. To determine the role of S100P in the regulation of cell cycle progression and cell proliferation, we investigated the effect on cell cycle progression and cell proliferation by over expression of S100P in ovarian cancer cell lines. The results showed that G2/M phase cells increased with a parallel decrease in G1-phase cells in S100P-overexpressed A2780 and SKOV3 cells. Consistently, over expression of S100P promoted ovarian cancer cell proliferation. These results fully demonstrated that S100P was involved in the regulation of cell cycle progression and cell proliferation. However, over expression of S100P didn't affect the migratory and invasive capability in A2780 and SKOV3 cells, suggesting that S100P may affect cell migratory and invasive capability in a cell/tissue specific manner. In future studies, we will further investigate the effect on cell migration and invasion by S100P using lentivirus-delivered stable gene silencing in ovarian cancer cells.

In conclusion, our findings suggest that S100P is an important molecular change significantly related to tumorigenesis and progression of

EOC. However, we need to further investigate the biological role and mechanism of S100P in EOC and extend studies about the role played by S100P in tumor growth and metastasis by using animal models. Importantly, a larger cohort of patients with ovarian cancer is still required to further define the clinical significance of S100P and its prognostic value in ovarian cancers in the future.

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Disclosure of conflict of interest

None.

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