

Decreased Eph receptor-A1 expression is related to grade in ovarian serous carcinoma

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Abstract. Eph receptor-A1 (EphA1) was the first member of the erythropoietin producing hepatocellular carcinoma (Eph) family of receptor tyrosine kinases. Although the roles of EphA1 in the tumorigenesis of various human cancers have been investigated, few studies have focused on ovarian carcinoma. The present study aimed to explore the profile of EphA1 expression in ovarian carcinomas, to analyze the association between EphA1 expression and clinicopathologic parameters, and to investigate the roles of overexpressed EphA1 in ovarian cancer cells. EphA1 protein was detected in ovarian cancer cell lines and in a set of formalin-fixed tissues, including normal fallopian tube, ovarian benign serous cystadenoma, borderline serous tumors and serous carcinoma. Ovarian cancer cell lines HO8910 and A2780 were transiently transfected with EphA1-pCMV6-GFP plasmid, and the proliferation and apoptosis of cells were measured. The association between EphA1 expression and clinicopathological parameters was statistically analyzed. EphA1 expression was negative in HO8910 and weakly positive in A2780 cells. The proliferation rate was significantly reduced in ovarian cancer cells after transfection with EphA1 plasmid compared with cells transfected with mock plasmid or untreated cells, but no obvious alteration in apoptosis was detected among these groups. EphA1 expression was positively detected in all normal fallopian tubes (10/10,

100%) and ovarian benign serous cystadenomas (12/12, 100%) as well as in some borderline serous tumors (9/15, 60%) and ovarian serous carcinomas (33/76, 43.42%). EphA1 expression was associated with grade of ovarian serous carcinomas, with loss of EphA1 more often observed in high-grade tumors (P=0.016) and high Ki67 index tumors (P=0.007). These data suggest that EphA1 might be a useful marker for distinguishing low grade from high-grade ovarian serous carcinoma.

Introduction

Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer, accounting for over 90% of ovarian cancers, and is one of the three most common cancers in females (1,2). EOC is associated with high morbidity and mortality rates owing to the typical late stage of the disease at diagnosis; up to 75% of females with EOC are diagnosed at advanced stages because there are few symptoms in the early stage (3). Ovarian serous carcinoma is the most common histological type of ovarian cancer, accounting for 70-80% of all newly diagnosed patients, and the most common and most aggressive subtype of EOC (4). Over the past 30 years, advances in surgery and chemotherapy have had little impact on overall patient survival, and current treatment leads to relapse in the majority of patients. This situation calls for investigation of the pathogenesis of ovarian serous carcinoma and identification of molecular markers for early diagnosis and treatment.

Erythropoietin producing hepatocellular carcinoma (Eph) receptors constitute the largest subfamily of receptor tyrosine kinases that bind membrane-bound ligands called ephrins (5). The Eph/ephrin interactions emanate their signals in a bidirectional manner into adjacent cells, followed by internalization and degradation of the complexes (6). Eph/ephrin signaling is proposed to participate in a wide spectrum of developmental processes through its capacity to regulate cellular adhesion, migration, or repulsion and tissue/cell boundary formation (7-11). Beyond their initial role in developmental processes, Ephs and ephrins are also involved in a broad range of processes directly related to tumor progression and metastasis (6,12-15).

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Eph receptor-A1 (EphA1), the first member of the Eph receptor tyrosine kinase family to be discovered, was isolated as a gene that was amplified in a carcinoma cell line and shown to be located on chromosome 7q34 (16). EphrinA1 is the highest affinity binding ligand for EphA1, although EphA1 also binds ephrinA3 and A4 with lower affinity. Whole-mount *in situ* hybridization showed overlapping expression of EphA1, ephrinA1, and ephrinA3 in the streak and the posterior paraxial mesoderm during early mouse development (17). Activation of EphA1 can inhibit cell spreading and migration in a Rho-ROCK-dependent manner (18). These results suggested that interaction of EphA1 and ephrinA1/A3 plays a role in tumor development. EphA1 expression has been detected in several types of human cancer. EphA1 mRNA and protein were detected in human epidermis at a high level, but EphA1 protein expression was reduced in non-melanoma skin cancers derived from the epidermis (19). In a previous study, we explored EphA1 expression in colorectal cancer, gastric cancer, and renal carcinoma and analyzed the correlation between EphA1 expression and clinicopathological parameters (20-22). Our data suggest that EphA1 is expressed in human cancers at highly varying levels. Expression of EphA1 protein has not yet been determined in ovarian serous carcinoma. The purpose of this study is to investigate the expression of EphA1 protein in ovarian serous adenocarcinoma and its association with clinical parameters.

Materials and methods

Cell lines and tissue samples. Human ovarian cancer cell lines HO8910 and A2780 used in the present study were purchased from the cell resource center of the Shanghai Institute of Life Sciences, Chinese Academy of Sciences. HO8910 and A2780 were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco; Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin in a 5% CO₂ and 95% atmosphere at 37°C with cell culture plates (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Clinical specimens were collected from the Department of Pathology of Affiliated Hospital of Nantong University from January 2001 to January 2013. Samples consisted of 10 normal fallopian tubes (age, 24-50 years; average, 43.1), 12 ovarian benign serous cystadenoma tumor tissues (age, 23-62 years; average, 38.5), 15 borderline serous tumors (age, 22-51 years; average, 34.5) and 76 ovarian serous carcinoma tissues (age, 27-69 years; average, 48.7). Generally, matched normal (or non-tumor) and tumor tissue from same patient are subjected to detection when we investigate a gene expression profile in certain cancer. However, serous ovarian carcinoma is very special. First of all, according to recent research results, serous ovarian carcinoma is not derived from ovarian epithelial cells, but from fallopian tube (23). Secondly, there usually almost no normal ovarian surface epithelial was available in ovarian tumor tissues. Formalin-fixed and paraffin-embedded tumor tissues were sectioned at 4- μ m thickness. Each tumor was classified according to WHO Classification Tumors of Female Reproduction Organs (23). Data were acquired with approval from the Ethics Committee of the Affiliated Hospital of Nantong University.

EphA1 plasmid transfection. The plasmid EphA1-pCMV6-GFP eukaryotic expression vector kit was purchased from OriGene Technologies, Inc (Rockville, MD, USA). HO8910 and A2780 cells were each divided into three groups: EphA1 transfected group (EphA1-TG), mock group (MG), and untransfected group (UTG). The MG group and the EphA1-TG group were transiently transfected with plasmid pCMV6-GFP and plasmid EphA1-pCMV6-GFP respectively using Lipofectamine 2000 according to the manufacturer's instructions. The UTG group did not receive any treatment. The transfection rate for EphA1-pCMV6-GFP and mock was checked by observation of GFP with a fluorescence microscope and by RT-PCR amplification of EphA1 mRNA. The protocol for amplification of EphA1 mRNA was the same as in our previous report (20). For EphA1, the sense primer is 5'-ATC TTTGGGCTGCTGCTTGG-3' and the antisense primer is 5'-GCTTGTCTCTCGATCCACATC-3'. For housekeeping gene GAPDH, the sense primer is 5'-CCAGGTGGTCTCCTC TGACTT-3' and the antisense primer is 5'-GTTGCTGTAGCC AAATTCGTTGT-3'.

Determination of cell viability (MTT assay). HO8910 and A2780 cells were seeded in 96-well flat-bottomed plates with 5,000 cells per well in 100 μ l of complete RPMI 1640 medium, followed by incubation at 37°C (5% CO₂ and 95% air) for 24 h to allow the cells to reach 70% confluence. The cells were transiently transfected and cultured for 48 h. The supernatant was carefully removed, and 100 μ l medium and 20 μ l of a 5 mg/ml MTT solution (Thermo Fisher Scientific, Inc.) were added to each well and incubated for 4 h at 37°C. The excess MTT was then aspirated. Viable cells internalize the MTT into their mitochondria. The formazan crystals formed in cells were dissolved by addition of 150 μ l of dimethyl sulfoxide (DMSO). After shaking for 1 h, the absorbance was measured at 540 nm in a multiwell scanning spectrophotometer.

Apoptosis. Apoptosis of cells after transfection for 72 h with EphA1-pCMV6-GFP and mock plasmid was detected using an Annexin V-FITC apoptosis detection kit (Qiagen GmbH, Hilden, Germany). In brief, cells were collected after digestion with 0.25% trypsin and rinsed. The cells were resuspended in binding buffer with 5 μ l Annexin V-FITC and 5 μ l propidium iodide (PI). After incubation at room temperature for 10 min in the dark, Annexin V-FITC/PI binding was measured by flow cytometry (excitation, 488 nm; emission, 530 nm) using the phycoerythrin emission signal detector (FL1 for detection of FITC, and FL2 for detection of PI).

Immunocytochemical (ICC) and immunohistochemical (IHC) staining. ICC and IHC staining was performed by the Envision method. For ICC staining, cells were grown on glass coverslips to 70% confluence, washed with PBS, and fixed with cold 75% ethanol for 10 min on ice. The cells were incubated in 3% H₂O₂ for 10 min and then at 4°C overnight with an anti-EphA1 polyclonal antibody (AO1047a, ABGENT) at a 1:100 dilution in Antibody Diluent (Zymed; Invitrogen). After a wash with PBS, the cells were incubated with secondary antibody (Dako, Ely, UK) for 20 min at room temperature. Color development was performed with 3,3'-diaminobenzidine (DAB). Nuclei were lightly counterstained with hematoxylin.

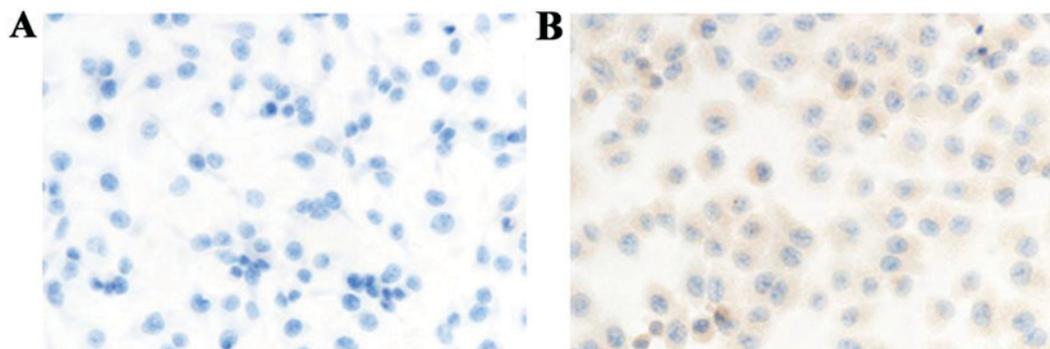


Figure 1. EphA1 expression was detected in ovarian cancer cell lines by immunocytochemical staining. (A) Expression of EphA1 protein was negative in the human ovarian cancer cell line HO8910. (B) Expression of EphA1 protein was weakly positive in the human ovarian cancer cell line A2780 (original magnification, x400). Eph1A1, Eph receptor-A1.

For IHC staining, 4- μ m thick sections were deparaffinized in xylene. After rehydration through a graded ethanol series, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval and then cooled to 30°C and washed with PBS (pH 7.3). After non-specific sites had been blocked with 3% H₂O₂ for 10 min, the sections were incubated at 4°C overnight with an anti-EphA1 polyclonal antibody and washed with PBS. The subsequent steps were the same as for ICC. Two pathologists independently assessed the immunostained slides, and any differences in the staining scores were resolved by consensus.

IHC scoring and quantification. Cytoplasmic staining was considered positive staining. The scoring for percentage of immunoreactive tumor cells was as follows: 0, 0%; 1, <20%; 2, 20-50%; and 3, >50%. The staining intensity was scored and stratified as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. A final immunoreactivity score (IRS) was obtained for each of the cases by multiplying the percentage score and the intensity score. Protein expression levels were further analyzed by classifying IRS values as negative (IRS value <4) or positive (IRS value \geq 4) (24).

Statistical analysis. Cell experiments were repeated three times and data were expressed as mean \pm standard deviation (mean \pm standard deviation). Results were analyzed by one-way ANOVA. The χ^2 test (Fisher's exact test) was used to assess the associations of EphA1 protein expression with clinicopathological variables. Two-sided P-values <0.05 were considered statistically significant. All analyses were performed by SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA).

Results

Expression of EphA1 in ovarian cancer cell lines. EphA1 expression in human ovarian cancer cell lines HO8910 and A2780 was examined by immunocytochemistry. EphA1 staining was located in the cytoplasm. The expression of EphA1 protein was negative in HO8910 cells and weakly positive in A2780 cells (Fig. 1).

Transfection of EphA1 gene. The ovarian cancer cells were observed using a fluorescence microscope after transient

transfection with pCMV6-GFP or EphA1-pCMV6-GFP plasmids. Green fluorescence was observed in ovarian cancer cells transfected with EphA1-TG and MG, but not in UTG (Fig. 2A). EphA1 mRNA expression in cells of the EphA1-TG group was detected by RT-PCR (127 bp), with GAPDH mRNA as an internal control (416 bp) (Fig. 2B).

Proliferation of HO8910 and A2780 cell lines after EphA1 transfection. The MTT assay was performed to determine the proliferative effect of HO8910 and A2780 ovarian cancer cells transfected with EphA1 and the data were analyzed using two-sample independent t-test. The proliferation rate of both HO8910-EphA1-TG and A2780-EphA1-TG cells was significantly reduced compared with that in mock and untransfected groups (Fig. 3).

Apoptosis in HO8910 and A2780 cell lines after EphA1 transfection. Apoptosis was measured in HO8910 and A2780 ovarian cancer cells using flow cytometry. There was no significant difference in apoptosis among the EphA1 transfected group, mock, and untransfected groups for both HO8910 and A2780 cells (Fig. 4).

EphA1 expression in normal fallopian tube and ovarian serous tumors. EphA1 staining in normal fallopian tube, ovarian benign serous cystadenoma, borderline serous tumors and serous carcinoma was located predominantly in the cytoplasm with diffuse positive expression (Fig. 5). Positive EphA1 staining was detected in all normal fallopian tubes (10/10) and ovarian benign serous cystadenomas (12/12). EphA1 protein was positively detected in some samples of borderline serous tumors (9/15) and ovarian serous carcinoma (33/76) (Table I).

EphA1 expression correlated with clinicopathological features. The relationship between EphA1 expression and clinicopathological parameters was shown in Table II. Statistical analysis of the association between EphA1 expression and clinicopathological features revealed a significant relationship between EphA1 expression and tumor grade (P=0.016) and Ki67 (P=0.007). No significant association of EphA1 expression and other features was found in this study.

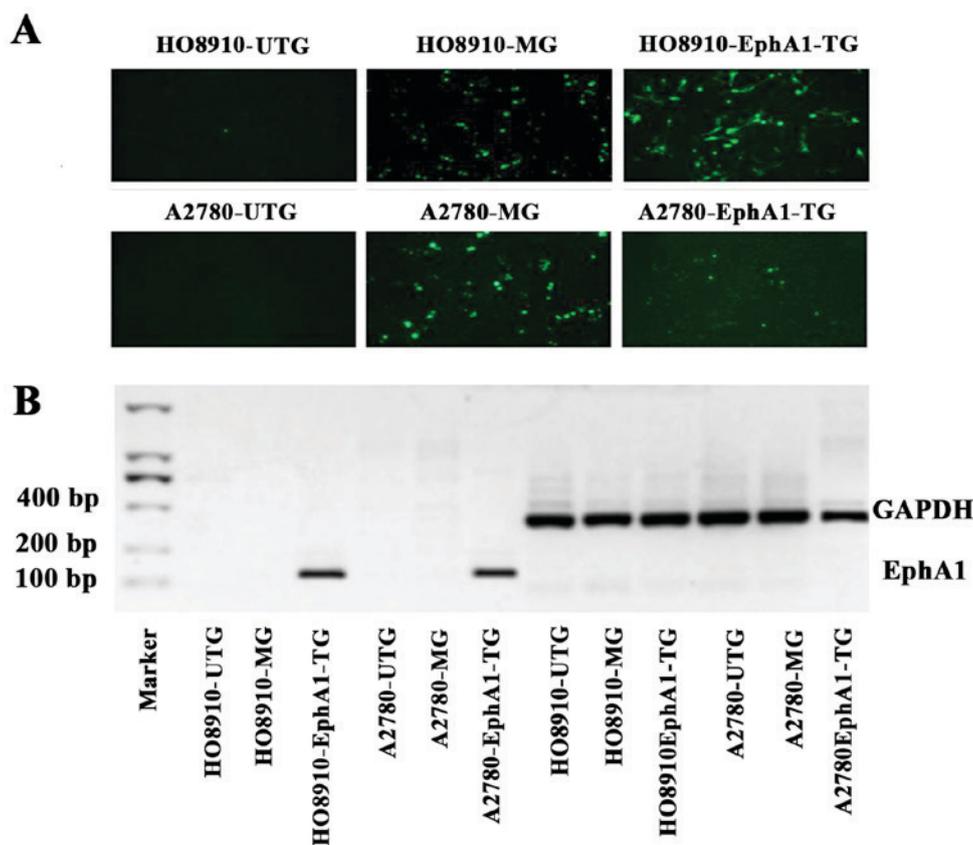


Figure 2. Transfection of EphA1-pCMV6-GFP and mock plasmid in ovarian cancer cell lines. (A) GFP signal was not observed in untransfected groups (HO8910-UTG and A2780-UTG); GFP signal was observed in mock groups (HO8910-MG and A2780-MG) and transfected groups (HO8910-EphA1-TG and A2780-EphA1-TG). (B) Transfection of EphA1 in ovarian cancer cell lines was confirmed by RT-PCR. EphA1 mRNA was amplified in transfected groups (HO8910-EphA1-TG and A2780-EphA1-TG), was not found in untransfected groups (HO8910-UTG and A2780-UTG) and mock groups (HO8910-MG and A2780-MG). EphA1, Eph receptor-A1; EphA1-TG, EphA1 transfected group; MG, mock group; UTG, untransfected group.

Table I. EphA1 expression in normal fallopian tubes, ovarian benign serous cystadenomas, and serous carcinomas.

Group	No.	EphA1		Positive (%)
		Negative	Positive	
Normal fallopian tube	10	0	10	100.00
Serous cystadenoma	12	0	12	100.00
Borderline serous tumors	15	6	9	60.00
Serous carcinoma	76	43	33	43.42

EphA1, Eph receptor-A1.

Discussion

Roles of the receptor tyrosine kinases in both normal physiology and oncogenesis have been well established. The genes that encode Eph receptors, the largest subfamily of receptor tyrosine kinases, are primarily considered to be classic oncogenes. Overexpression of EphA1 has been reported in several human cancers (25-27); however, reduced expression of EphA1 also has been detected in prostate cancer cell lines (28), basal

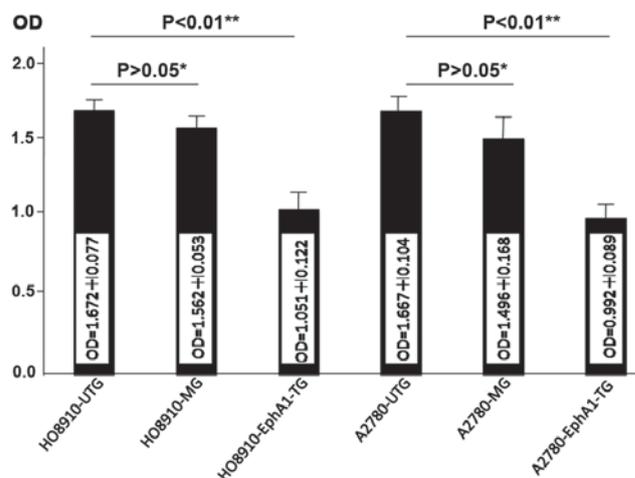


Figure 3 The MTT assay was performed to determine the proliferative effect of HO8910 and A2780 ovarian cancer cells transfected with EphA1. No significant difference was observed in UTG and MG groups of both HO8910 and A2780 ($P>0.05$). The proliferation rate in transfected groups (HO8910-EphA1-TG and A2780-EphA1-TG) was significantly reduced compared with MG ($P<0.01$) and UTG ($P<0.01$). EphA1, Eph receptor-A1; EphA1-TG, EphA1 transfected group; MG, mock group; UTG, untransfected group.

cell carcinomas and squamous cell carcinomas of the skin (19), and colorectal cancer (20). Therefore, whether EphA1 is an oncogene has been questioned. We previously reported that

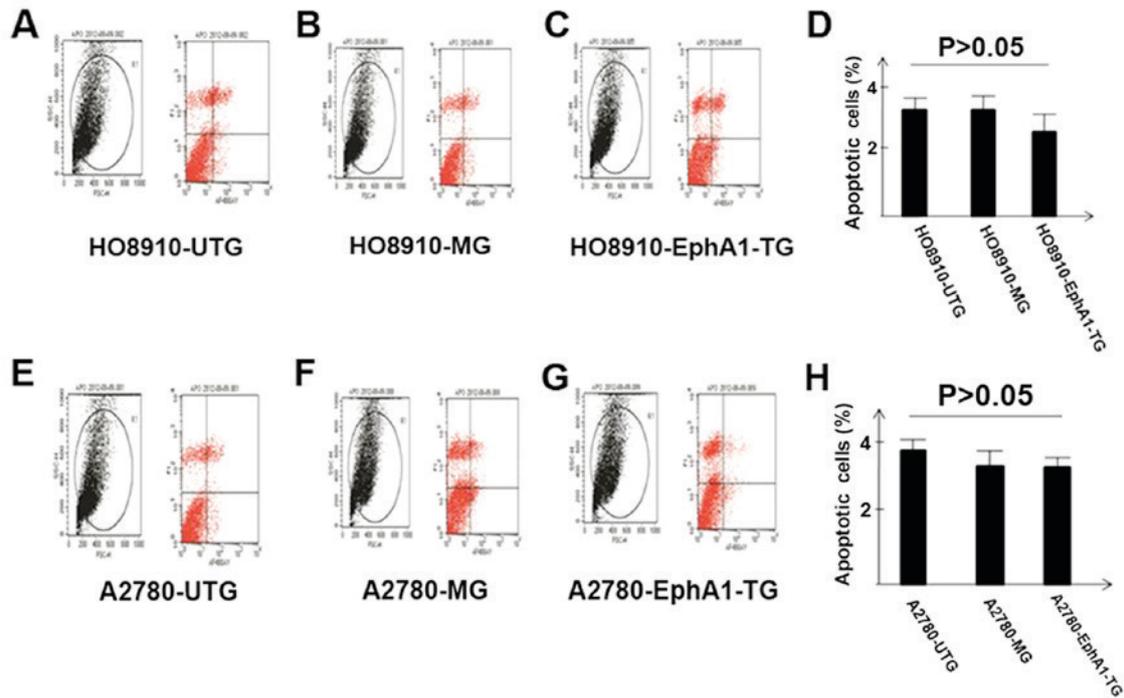


Figure 4. Apoptosis was measured in HO8910 and A2780 ovarian cancer cells using flow cytometry. There was no significant difference in apoptosis among the UTG (A and E), MG (B and F), and EphA1-TG (C and G) in both HO8910 (D; $P>0.05$) and A2780 cells (H; $P>0.05$). Eph1A1, Eph receptor-A1; EphA1-TG, EphA1 transfected group; MG, mock group; UTG, untransfected group.

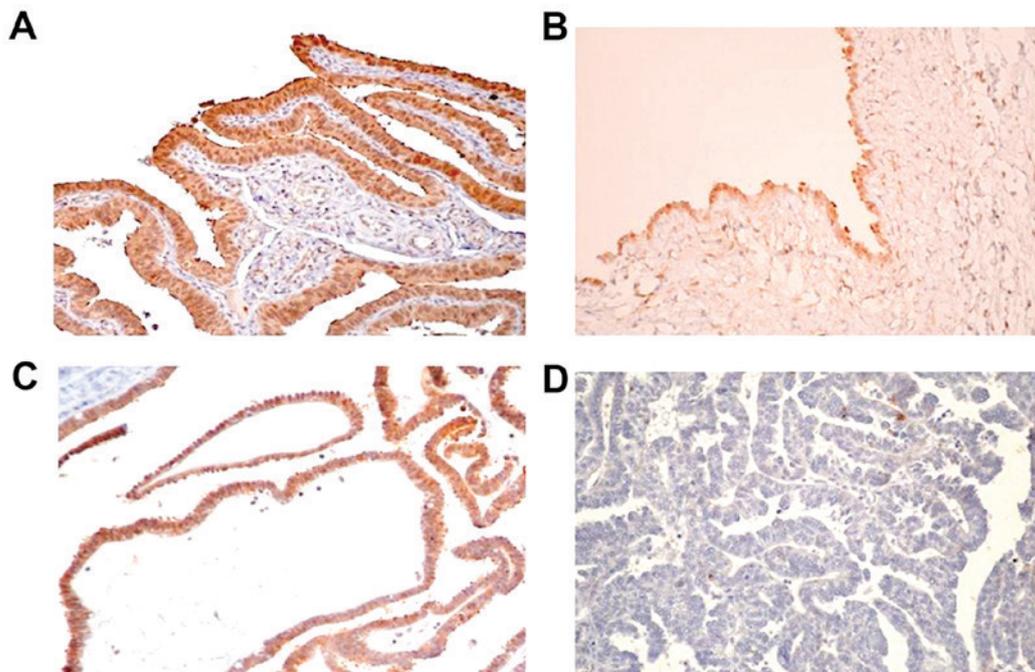


Figure 5. EphA1 expression was detected in ovarian tissues by immunohistochemistry. EphA1 expression was positive in normal fallopian tube (A), benign serous cystadenoma (B), and low-grade ovarian serous carcinoma (C) and negative in high-grade ovarian serous carcinoma (D). Original magnification, x400. Eph1A1, Eph receptor-A1.

EphA1 expression was associated with metastasis in esophageal squamous cell carcinoma (29), Gleason score in prostate cancer (30), invasion and metastasis in colorectal cancer (20), and metastasis in gastric cancer (21). Comprehensive studies show obvious differences in EphA1 expression among different tissues and different tumor types.

In this study, EphA1 expression was negative in HO8910 and weakly positive in A2780 ovarian cancer cells. In addition, loss of EphA1 expression was found in most ovarian serous carcinoma tissues compared with normal fallopian tube and benign tumor. Our data suggest that EphA1 is downregulated in ovarian serous carcinoma. In contrast, Wong *et al* reported

Table II. Relationship between EphA1 expression and clinicopathologic parameters in ovarian serous carcinomas.

Parameters	No.	EphA1		Positive %	χ^2	P-value
		-	+			
Clinical stages						
I+II	15	6	9	60.00	2.091	0.148
III+ IV	61	37	24	39.34		
Grade						
Low	12	3	9	75.00	5.784	0.016
High	64	40	24	37.50		
Metastasis						
Yes	30	19	11	36.67	0.920	0.337
No	46	24	22	47.83		
Position of tumors						
Single	32	16	16	50.00	0.974	0.324
Double	44	27	17	38.64		
Maximum diameter (cm)						
≤5	29	13	16	55.17	3.938	0.140
5-10	21	15	6	28.57		
≥10	17	11	6	35.29		
No data	9	4	5			
Age (years)						
≤50	30	17	13	43.33	3.410	0.182
50-55	16	12	4	25.00		
≥55	30	14	16	53.33		
Ki67						
>20%	52	35	17	68.42	7.715	0.007
≤20%	24	8	16	31.58		

Eph1A1, Eph receptor-A1.

that EphA1 mRNA was upregulated in EOC with positive immunostaining of ephrin receptor A1 (31) and Herath *et al* reported that overexpression of EphA1 mRNA strongly correlated with the high-affinity ligand ephrin A1 in advanced ovarian cancer (32). We interpret the difference in results between these papers and ours as follows: First, the experimental samples used by Herath *et al* (32) and Wong *et al* (31) included ovarian serous, mucinous, endometrioid, and clear cell cancer, whereas we focused on ovarian serous cancer. Second, mechanisms regulating EphA1 protein level may be important in ovarian cancer and EphA1 mRNA expression may show an inconsistent trend. The possible mechanisms need to be further explored. We previously proved that hypermethylation of a CpG island in the EphA1 promoter region leads to downregulation of EphA1 in colorectal cancer (20). We therefore deduced that methylation of DNA might be one of the mechanisms for reduced expression of EphA1 in ovarian serous cancers. Other possible regulatory mechanisms include EphA1 mutation, microRNA, deacetylation, and gene

deletion. We plan to intensively investigate these molecular mechanisms in future studies.

Ki67 is a marker of proliferation expressed exclusively during active phases of the cell cycle. It is commonly assessed by IHC in clinical settings to judge cell proliferative activity. It has been correlated with clinical outcome and is considered to be an indicator of prognosis. Interestingly, our data show that loss of EphA1 was more often observed in high Ki67 index tumors (P=0.007). On the other hand, the MTT proliferation assay showed that overexpression of EphA1 gene inhibited the proliferation of HO8910 and A2780 tumor cells, this is consistent with what observed in tumor tissues. Overexpression of EphA1 in ovarian cancer cell lines did not affect cell apoptosis. Our results suggest that EphA1 may play a role in ovarian cancer as a tumor suppressor but is not a key suppressor gene in ovarian tumorigenesis.

Histologic grade has been shown to be an important prognostic factor in cases of ovarian serous carcinoma. Although the ovarian grading system has evolved over the years, there is no universally accepted classification. The Federation of Gynecology and Obstetrics (FIGO) grading system typically analyzes architectural pattern, nuclear/cytologic atypia, mitotic index, or a combination of these features. Molecular pathological research has contributed to improved knowledge of the different subtypes of ovarian cancer. The World Health Organization Classification System of Ovarian Cancer, published in 2014 by Kurman *et al* (23), eliminated the older practice of grading serous tumors on a continuum (grade 1, 2, or 3) and instead differentiates low-grade serous and high-grade serous ovarian cancers as two distinct diseases. It is now widely accepted that low-grade and high-grade serous tumors are essentially distinct diseases exhibiting distinct genetic alterations, molecular patterns, and clinical behaviors. Low-grade serous carcinoma develops from well-recognized precursors and behaves in an indolent fashion. It is characterized by specific mutations including KRAS, BRAF, and ERBB2 and is relatively genetically stable (1). In contrast, high-grade serous ovarian carcinoma is characterized by advanced stage at diagnosis, frequent TP53 mutation, rapid progression, and high responsiveness to platinum-based chemotherapy (33). Although high-grade and low-grade serous carcinomas are usually easily distinguished, it may be difficult to discriminate between them in some carcinomas and can especially challenging in small tissue samples (34). This is the first study demonstrating that EphA1 protein is significantly correlated with tumor grade in ovarian serous carcinoma, with negative expression of EphA1 more often found in ovarian high-grade serous cancers (P=0.016). Our data suggest that EphA1 may be a new molecular marker for grading ovarian serous carcinoma.

In conclusion, EphA1 expression is decreased in ovarian serous carcinoma compared with normal fallopian tube and benign ovarian serous cystadenoma. Decreased EphA1 expression was more often detected in high-grade tumors. Our data suggest that EphA1 may be a new marker for grading and prognosis in ovarian serous adenocarcinoma.

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