Semaphorin-4C is upregulated in epithelial ovarian cancer

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Abstract. The present retrospective study aimed to investigate the expression of semaphorin-4C (Sema4C) in epithelial ovarian cancer (EOC), and to determine the association between Sema4C expression and patient clinicopathological characteristics. Sema4C mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction in the tissues of 74 cases of EOC, 20 cases of ovarian epithelial benign tumor, 20 cases of ovarian borderline epithelial tumor and 15 cases of normal ovarian tissue. Immunohistochemistry was used to detect the expression and localization of Sema4C. The association between Sema4C expression level and patients clinicopathological characteristics was determined by χ^2 test. The results demonstrated that Sema4C expression level in ovarian epithelial carcinoma tissues was significantly higher compared with that in benign tumors, borderline epithelial tumors and normal ovarian tissues ($P<0.05$). In addition, Sema4C expression in ovarian cancer tissues was significantly associated with the clinical and pathological stages of tumors ($P<0.05$). In conclusion, the present study demonstrated that Sema4C expression was upregulated in EOC.

Introduction

Ovarian cancer is a common gynecological malignancy that has the highest mortality rate among all cancer types affecting women worldwide, with >200,000 new cases each year (1). Ovarian cancers are histologically defined as type I or type II. Type I refers to a relatively low histological grade, including endometrioid, mucinous and clear-cell carcinomas, whereas type II refers to a higher histological grade, including serous carcinoma and carcinosarcoma (2). Epithelial ovarian cancer (EOC) is the main type of ovarian cancer and represents >90% cases. In addition, ~90% of patients dying from EOC suffer from type II EOC (3). Treatment for ovarian cancer usually includes a combination of surgery, radiation therapy and chemotherapy (4). However, the outcome of patients depends also on their clinicopathological characteristics, including the subtype of ovarian cancer and the presence of other medical conditions (5). In addition to the aforementioned conventional treatments, targeted therapy, also known as molecularly targeted therapy, is one of the major treatment options, which only targets cancer cells (6). Targeted therapy may therefore be considered as a promising cure for patients with ovarian cancer in the near future.

Semaphorins are members of a family of membrane-bound and secreted molecules, which were originally identified as evolutionarily conserved axon-guidance cues in the human neural circuitry (7,8). The semaphorin family is divided into eight classes, which consist of >30 genes, while the number of semaphorins is still rising. The neuropilin and plexin gene families encode the main semaphorin receptors (9). It has been widely reported that semaphorins are highly expressed in the human nervous system. For example, previous studies demonstrated that certain semaphorin members, including semaphorins 6B and 5B, are involved in the progression of various types of cancer, including gastric cancer (10) and renal cell carcinoma (11). These semaphorins promote the progression and angiogenesis of tumor cells via numerous mechanisms, including the modulation of tumor angiogenesis (10,11). Furthermore, certain semaphorins, including class 3 semaphorins, have been reported to inhibit tumor progression, whereas others, inducing semaphoring 4D, were demonstrated to promote tumor progression (9). To the best of our knowledge, there is no study about the expression of semaphorin-4C (Sema4C) in EOC.

Therefore, the present study investigated the expression of Sema4C in EOC and determined its association with the clinicopathological characteristics of patients with EOC.

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Materials and methods

Patients and tumor samples. EOC cancer tissues were obtained from patients who were surgically treated at the Department of Oncology of Yantaishan Hospital (Yantai, China) between January 2013 and January 2018. Cancer tissues were obtained within 30 min of the resection, placed in liquid nitrogen and stored at -80˚C. In total, 74 cases of EOC, 20 cases of ovarian epithelial benign tumor, 20 cases of ovarian borderline epithelial tumor and 15 cases of normal ovarian tissues were collected. The age distribution of patients with EOC was 29-67 years (mean age, 51.2±7.6 years). The age distribution of patients with ovarian epithelial benign tumor was 30-68 years (mean age, 47.2±7.7 years). The age distribution of patients with ovarian borderline epithelial tumor was 28-61 years (mean age, 43.6±6.8 years). The age distribution of patients with normal ovarian tissues was 30-63 years (mean age, 45.6±7.4 years). The patients had no heart-, liver-, lung-, kidney- or other important organ-related diseases, and had no history of chemotherapy, radiotherapy or other treatment prior to surgery. Patients with other malignant tumors were also excluded.

The 74 cases with EOC were graded according to the World Health Organization (WHO) standards for histopathological clinical staging (12) as follows: A total of 44 patients had stages I and II EOC, whereas 30 patients had stage III EOC. However, according to the Union for International Cancer Control (UICC) standards (13), 21 cases were in stages I-II, whereas 53 cases were in stages III-IV. In total, 55 patients out of the 74 cases were >50 years old, and 49 out of the 74 patients presented with ascites.

EOC cancer tissues were obtained from patients who were surgically treated at the Department of Oncology of Yantaishan Hospital (Yantai, China) between January 2013 and January 2018 and who were diagnosed with EOC. The tissues previously mentioned were part of these tissues. In total, 111 EOC tissues were collected and embedded in paraffin before analyzing Sema4C protein expression. According to the WHO standards for histopathological clinical staging, 69 cases were in stages I and II, whereas 44 cases were in stage III. However, according to the UICC standards, 29 cases were in stages I-II, whereas 82 cases were in stages III-IV. In total, 84 patients were >50 years old and 75 patients presented with ascites. The clinical data of all the patients were complete, and the pathological data were provided by a physician in-chief from the Pathology department of Yantaishan Hospital.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and RNA purity was determined by calculating the 260/280 ratio of optical densities using a nucleic acid-protein detector (DU-640; Beckman Coulter, Inc.). The result was between 1.8 and 2.0, which indicated sufficient RNA purity. cDNA was synthesized using an Eppendorf PCR Mastercycler (Eppendorf) according to the manufacturer's instructions, whereas qPCR was performed using SYBR-Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the GeneAmp 5700 Sequence Detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCRs were performed as follows: 94˚C, melting under pre-denaturation for 5 min; 94˚C for additional 30 sec, 72˚C for 45 sec and 62˚C for 30 sec (all steps were repeated for 35 cycles); and maintenance at 72˚C for 10 min. The primer sequences for Sema4C were synthesized by Shanghai GenePharma Co., Ltd. and were as follows: Sema4C, forward, 5'-ACCTTGTGCCGCGTAAGACAG-3' and reverse, 5'-CGTCAGCGTCAGTGTCAGGAA-3'; and β-actin, forward, 5'-CCTGGGCATGGAGTCCTGTG-3' and reverse, 5'-AGGGGCCGGACTCGTCATAC-3'. The relative expressions level of Sema4C was normalized to the endogenous control β-actin and was expressed as $2^{-\Delta\Delta Cq}$ (14).

Immunohistochemistry (IHC) staining. Tissue sections from paraffin-embedded cancer tissues were incubated at 60˚C for 30 min, dewaxed using xylene and rehydrated using a decreasing ethanol gradient (100, 95, 75 and 50%, 5 min each time). Sections were washed three times for 5 min with PBS. Sections were incubated in 3% H_2O_2 dissolved in 80% methanol at room temperature for 10 min to inactivate endogenous peroxidase. Tissues were heated at 95˚C for 20 min and blocked with 5% bovine serum albumin (cat. no. B2064; Sigma-Aldrich; Merck KGaA) for 20 min. Tissues were then incubated with rabbit polyclonal human primary antibody against Sema4C (1:400; cat. no. PA5-52788; Thermo Fisher Scientific, Inc.) at 4˚C overnight, and incubated with goat anti-rabbit IgG secondary antibody (1:1,000; MH1732; Thermo Fisher Scientific, Inc.) at 37˚C for 20-30 min. Signals were visualized using 3'-diaminobenzidine staining (cat. no. TA-060-QHDX; Thermo Fisher Scientific, Inc.) at 37˚C for 5-10 min and hematoxylin counterstained at 37˚C for 30 sec-1 min. Differentiation was induced by hydrochloric acid and ethanol dehydration (80, 95 and 100%, 5 min each time). For each slice, images of 10 sections were acquired under an optical microscope (BX45-72H05; Olympus Corporation; magnification, x100) to count positively stained cells. A percentage of positively stained cells >30% was considered as a positive staining.

Statistical analysis. SPSS version 13.0 statistical software (SPSS Inc.) was used to statistically analyze the data. The results were expressed as the means ± standard deviation. The t-test was used for comparisons between two datasets, whereas one-way analysis of variance followed by least-significant difference post hoc test was used for comparisons among multiple datasets. $P<0.05$ was considered to indicate a statistically significant difference. The Sema4C protein expression levels were compared between groups using χ^2 test. The correction between Sema4C mRNA level and Sema4C protein expression were analyzed by Pearson's correlation analysis.

Results

Sema4C is upregulated in EOC tissues. The results from RT-qPCR demonstrated that Sema4C expression level was significantly higher in malignant tissues compared with that in borderline, benign and normal tissues ($P<0.001$; Table I). In addition, the 74 cases of EOC were divided into four groups as follows: Serous carcinoma, mucinous adenocarcinoma, endometrial cancer uterus and clear cell carcinoma (Table I).

Table I. Sema4C mRNA expression in different ovarian tissues.

Variable	Number	Sema4C mRNA ($\bar{x}$ ± SD)	T-value	P-value
Category				
Malignant	74	0.0505±0.0308	34.193	<0.001
Borderline	20	0.0074±0.0113		
Benign	20	0.0067±0.0082		
Normal	15	0.0059±0.0072		
Histological type				
Serous carcinoma	24	0.0440±0.0212	1.012	>0.05
Mucinous adenocarcinoma	21	0.0595±0.0444		
Endometrial cancer uterus	15	0.0497±0.0295		
Clear cell carcinoma	14	0.0501±0.0282		

SD, standard deviation; Sema4C, semaphorin-4C.

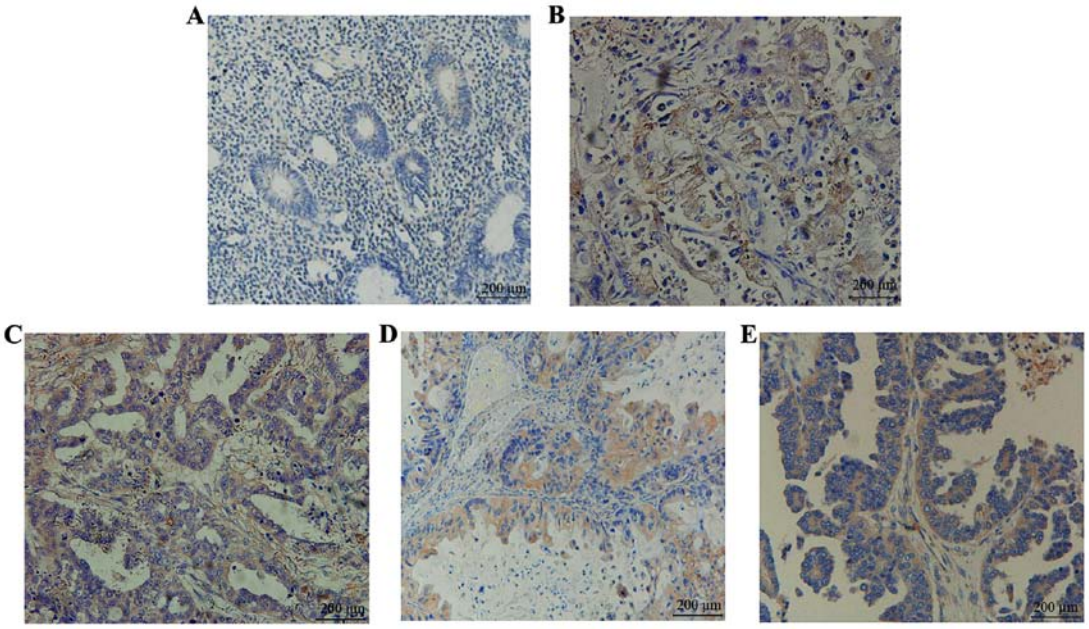

Figure 1. Immunohistochemical staining of ovarian tissues. Representative images of (A) normal ovarian tissue, (B) ovarian clear cell carcinoma tissue, (C) endometrioid ovarian cancer tissue, (D) mucinous ovarian cancer tissue and (E) serous ovarian cancer tissue. Magnification, x100.

These results indicated that Sema4C was highly expressed in all cancer tissues, but its expression level was not associated with the histological type (P>0.05).

To examine the expression of Sema4C protein in ovarian cancer, IHC was used. The results demonstrated that Sema4C protein was hardly expressed in the normal ovarian tissue (Fig. 1A), whereas it was highly expressed in EOC tissues (Fig. 1B-E). These findings confirmed that Sema4C mRNA and protein expression were highly expressed in EOC tissues.

Sema4C expression is associated with differentiation and clinical stage of EOC tissues. The association between Sema4C mRNA expression and numerous factors was analyzed, including cancer differentiation level, clinical stage, EOC ascites and age of the patients at disease onset. The results demonstrated that Sema4C mRNA expression was significantly higher in the medium/high differentiation group compared with that in the low differentiation group (P=0.011; Table II). Furthermore, Sema4C mRNA expression in stages III and IV of ovarian cancer was significantly higher than that in stages I and II (P=0.014). These results indicated that Sema4C mRNA expression in EOC was associated with tissue differentiation, FIGO stage and ascites.

Furthermore, the association between Sema4C protein and clinicopathological factors was investigated, including histological type, ascites, age, differentiation and FIGO stage (Table III). The results demonstrated that Sema4C protein positive expression in the medium/highly-differentiated group (75.0%) was significantly higher compared with that in the low differentiation group (52.2%; P=0.016). In addition, Sema4C protein positive expression in tissues at clinical stages III-IV (68.3%) was also significantly increased compared with that at

Table II. Association between Sema4C mRNA expression and clinicopathological features of patients with epithelial ovarian cancer.

Variable	Number	Sema4C mRNA ($\bar{x}$ ± SD)	T-value	P-value
Differentiation				
Low	44	0.0431±0.0238	2.598	0.011
Medium/high	30	0.0614±0.0367		
FIGO stage				
I-II	21	0.0367±0.0236	2.527	0.014
III-IV	53	0.0560±0.0318		
Ascites				
Yes	49	0.0536±0.0331	1.213	0.229
No	25	0.0445±0.0253		
Age, years				
<50	19	0.0467±0.0279	-6.332	0.529
≥50	55	0.0519±0.0319		

SD, standard deviation; Sema4C, semaphorin-4C; FIGO, International Federation of Gynecology and Obstetrics.

Table III. Association between semaphoring-4C protein expression and clinicopathological characteristics of patients with ovarian cancer.

Variable	Positive, n (%)	Negative, n (%)	χ^2	P-value
Histological type				
Serous carcinoma	16 (53.3)	14 (46.7)	2.907	0.406
Mucinous adenocarcinoma	22 (73.3)	8 (26.7)		
Endometrial cancer uterus	17 (56.7)	13 (43.3)		
Clear cell carcinoma	13 (61.9)	8 (38.1)		
Ascites				
Yes	49 (65.3)	26 (34.7)	1.616	0.204
No	19 (52.8)	17 (47.2)		
Age, years				
<50	14 (56.0)	11 (44.0)	0.376	0.540
≥50	54 (62.8)	32 (37.2)		
Differentiation				
Low	35 (52.2)	32 (47.8)	5.798	0.016
Medium/high	33 (75.0)	11 (25.0)		
FIGO stage				
I-II	12 (41.4)	17 (58.6)	6.539	0.011
III-IV	56 (68.3)	26 (31.7)		

FIGO, international federation of gynecology and obstetrics.

clinical stages I-II (41.4%; P=0.011). These findings indicated that Sema4C protein expression was associated with the differentiation and FIGO stage of EOC, but not with histological type, ascites and age (P>0.05).

Sema4C is upregulated in late-stage EOC. The positive expression rate of Sema4C in EOC tissues was 61.3% (Table IV), whereas the values for borderline ovarian epithelial tumor, benign tumor and normal ovarian tissues were 26.7, 16.7 and 10.0%, respectively. The results of χ^2 test demonstrated that the positive expression rate of Sema4C in EOC tissues was significantly higher compared with that in the other three types of tissue (P<0.01; Table IV).

The results from Pearson's correlation analysis (Table V) revealed that Sema4C mRNA expression and Sema4C protein expression in EOC tissues were positively correlated (P<0.01). The regression equation was Y=-1.50814+1.052126X, with a correlation coefficient of R^2=0.955 (P<0.01). Furthermore,

Table IV. Expression of semaphorin-4C protein in different categories of ovarian tissue.

Category	Positive, n (%)	Negative, n (%)	χ^2	P-value
Malignant	68 (61.3)	43 (38.7)	40.367	<0.01
Borderline	8 (26.7)	22 (38.7)		
Benign	5 (16.7)	25 (83.3)		
Normal	3 (10.0)	27 (90.0)		

Table V. Pearson's correlation analysis between the mRNA and protein expression of Sema4C.

Sema4C expression	Positive, n (%)	Negative, n (%)	R^2	P-value
mRNA	44 (59.46)	30 (40.54)	0.955	<0.001
Protein	68 (61.26)	43 (38.74)		

Sema4C, semaphorin-4C.

the results from Pearson's correlation analysis revealed that Sema4C mRNA expression and Sema4C positive expression rate were positively correlated with tumor malignancy and clinical stage.

Discussion

Semaphorins were originally reported as serving crucial role in nervous system (10,15,16). Over the past decade, semaphorins have been thought to be involved in numerous developmental processes, including cell migration and invasion (15-18). In particular, Sema3B and Sema3F were successfully identified as modulators of tumor progression (17,18). In addition to these two semaphorins, semaphorins 6B has been characterized as regulators of tumor progression (10). To the best of our knowledge, the present study was the first to confirm that both Sema4C mRNA and protein expression were highly expressed in EOC tissues. Furthermore, Sema4C mRNA expression in EOC was associated with tissue differentiation, FIGO stage and ascites. Sema4C protein expression was also found to be upregulated in late-stage EOC.

Class 4 semaphorins are single-pass transmembrane proteins that usually exert clear influences on tumor progression. For example, Sema4D was demonstrated to be upregulated in several types of cancer, including head and neck, cervical, colon, prostate, lung and breast cancer (19). In addition, Sema4C is expressed at a relatively low rate (3.3%), or not at all in normal ovarian tissues, which was similar to the findings of the present study. In the present study, Sema4C protein was expressed at a low rate (3.0%) in normal ovary. In addition, Sema4C protein was positively expressed in EOC (56.0%), and was mostly located in the cytoplasm and/or cell membrane.

A previous study reported that Sema4C stimulates the production of angiogenin and colony-stimulating factor-1 in breast cancer cells by activating the NF-κB signaling pathway (20). Furthermore, Gurrapu *et al* (21) reported that Sema4C/PlexinB2 signaling pathway was essential for breast carcinoma cell proliferation, suggesting that it might be considered as a novel potential therapeutic target. In addition, it was reported that elevated Sema4C expression enables indolent luminal-type tumors to become resistant to estrogen deprivation, invasive and metastatic *in vivo*. The present study reported that Sema4C was highly expressed in ovarian epithelial cancer tissues; however, the underlying mechanisms remain clear. The role of Sema4C in the stimulation of ovarian epithelial cancer growth requires therefore further investigation.

In conclusion, the present study demonstrated that Sema4C was highly expressed in EOC tissues, and that Sema4C mRNA and protein expression were associated with tumor malignancy and clinical stage. These findings suggested that high Sema4C expression in EOC tissues may be associated with poor prognosis in patients with EOC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SYH, SH and JW participated in the design of the study. SYH, SH, JZ and ZZ carried out RT-qPCR and IHC experiments and performed statistical analysis. SYH drafted the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Yantaishan Hospital and all patients provided written informed consent (clinical trial no. ChiCTR1900020785).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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