RESEARCH ARTICLE

Overexpression of Semaphorin4D Indicates Poor Prognosis and Prompts Monocyte Differentiation toward M2 Macrophages in Epithelial Ovarian Cancer

Ying Chen[1,3,4&*], Lei Zhang[1,3,4&], Rui Lv[2], Wen-Qi Zhang[1,3]

Abstract

Previously, we demonstrated overexpression of semaphorin4D (SEMA4D, CD100) to be closely related to tumor angiogenesis in epithelial ovarian cancers (EOCs). However, the function and expression of SEMA4D in the EOC microenvironment has yet to be clarified in detail. In this study, we confirmed that overexpression of SEMA4D in primary tumors and ascites was related to low differentiation, platinum resistance and a refractory status ($P<0.05$), while high M2 macrophage count and percentage were evident in EOC patients with advanced FIGO stage and platinum resistance ($P<0.05$), using immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and fluorescence-activated cell sorting (FACS), respectively. The data showed correlations of SEMA4D expression and M2 macrophage counts in primary tumors and M2 macrophage percentage in ascites (r=0.281 and 0.355, each $P<0.05$). In the Cox proportional hazard mode, SEMA4D expression was an independent indicator of overall survival (OS) and progression-free survival (PFS) for EOC patients. Furthermore, higher expression of SEMA4D in ovarian cancer cell lines (SKOV3, A2780, and SW626) and their supernatants were found than that in a human primary cultured ovarian cell and its supernatant by reversed transcript PCR (RT-PCR), Western blotting and ELISA, respectively. Interestingly, peripheral blood monocytes (MOs) tended towards the M2-polarized macrophage phenotype ($CD163^{high}$) *in vitro* after human recombined soluble SEMA4D protein stimulation. These findings suggest that SEMA4D might possibly serve as a reliable tool for early and accurate prediction of EOC poor prognosis and could playan important role in promoting tumor dissemination and metastasis in the EOC microenvironment. Thus SEMA4D and its role in macrophage polarization in EOC warrants further study.

Keywords: Semaphorin4D - ascites - macrophage - ovarian cancer - prognosis

Asian Pac J Cancer Prev, **14 (10)**, 5883-5890

Introduction

Epithelial ovarian cancer (EOC) is the leading killer among all gynecological malignancies (Siegel et al., 2012). Over 70% of EOC patients present with late stage diseases and dissemination of tumor implants throughout the peritoneal cavity (Bast et al., 2009). Ascites is one of the most common and distressing complications associated with EOC, especially for advanced stage diseases (Auersperg et al., 2002). Different from stroma surrounding solid tumors, ascites constitute a unique form of tumor microenvironment. Recent evidences suggested that ascites of EOC play a positive role on cancer peritoneal dissection and metastasis owing to containing plenty of growth factors, cytokines, extracellular matrix constituents, and cancer-promoting immunocytes, especially tumor-associated macrophages (TAMs), which induced tumorigenesis closely (Abdollahi et al., 2003; Martinez et al., 2008; Giuntoli et al., 2009). Macrophages can be phenotypically polarized by the microenvironment to mount specific functional programs, including classically activated macrophages (M1) and alternatively activated macrophages (M2), the latter demonstrating pro-tumor functions and specific expressing scavenger receptor CD163 (Qian et al., 2010). Macrophages in the tumor microenvironment are defined as TAMs and chiefly exhibit M2 characteristics. Importantly, collective data proved high numbers of "alternatively activated" TAMs or M2 macrophages are associated with a worse prognosis in numerous cancer types (Lee et al., 2008; Jensen et al., 2009; van Dongen et al., 2010).

Semaphorin4D (SEMA4D, also known as CD100), one of the transmembrane or secreted Semaphorin proteins IV subfamily member, originally was identified for its activity

[1]Department of Gynecologic Oncology, Tianjin Medical University Cancer Institute and Hospital, [2]Reproductive Medical Center, Tianjin Medical University General Hospital, [3]Key Laboratory of Cancer Prevention and Therapy, Tianjin, China, [4]National Clinical Research Centre of Cancer [&]Equal contributors [*]For correspondence: lychenying2004@126.com

in the nervous system (Sakurai et al., 2012). Recently, cumulative evidences showed that the soluble form of SEMA4D can promote many biologic effects observed in immune system and tumor environment, especially playing a role in monocytes and macrophages (MO/MAs) activation (Kumanogoh et al., 2004; Basile et al., 2006). Ishida et al. reported that soluble human SEMA4D could induce the production of pro-inflammatory cytokines by MO/MAs, including IL-6 and IL-8 (Ishida et al., 2003). Significantly, previous study proved elevated IL-6 in ascites suggested a shorter progression-free survival (PFS) of EOC patients (Lane et al., 2011) and EOC cell invasion and migration were accelerated due to IL-8 over-expression (Zhang et al., 2010). Delaire S et al. had also reported that human soluble SEMA4D inhibits both the spontaneous and induced migration of MO/MAs in tumor microenvironments (Delaire et al., 2001). Moreover, former data demonstrated soluble SEMA4D protein released from the breast cancer cell surface acted on local as well as distant tumor microenvironments, thus inducing tumor angiogenesis and metastasis (Basile et al., 2007). Furthermore, the same authors showed SEMA4D produced by TAMs not tumor cells per se enhanced tumor growth and angiogenesis in head and neck squamous cell carcinoma (HNSCC) (Sierra et al., 2008). The authors explained the seemingly contradictory results possibly due to differences between these tumor cell lines.

Indeed, SEMA4D had been verified highly expression in a wide range of human tumors such as prostate, colon, breast, oral, HNSCC, and soft tissue sarcomas (Basile et al., 2006; Ch'Ng et al., 2007). Previously, we also demonstrated over-expression of SEMA4D was closely related to tumor angiogenesis in EOC tissues (Chen et al., 2012). However, the function and expression of SEMA4D in EOC microenvironment has not yet been clarified. In this study, we focus on detecting the expressions of SEMA4D and M2 macrophage in primary tumor tissues and ascites of EOC patients and assess their association with the established clinicopathologic factors of the disease as well as patient prognosis. Consequently, the expressions of SEMA4D in ovarian cancer cell lines (SKOV3, SW626, and A2780) and human primary cultured ovarian cell and their supernatants were evaluated by enzyme-linked immunosorbent assay (ELISA), Reversed Transcript PCR (RT-PCR), and Western blot, respectively. And then, phenotypes of MOs (from healthy donors) stimulated with/without human recombined soluble SEMA4D protein and macrophages (from EOC ascites) were investigated by Fluorescence-activated cell sorting (FACS).

Materials and Methods

Patients and specimens

The study consisted of 67 primary EOC patients with ascites no less than 200 ml, diagnosed and treated at the Department of Gynecologic Oncology, Tianjin Medical University Cancer Institute and Hospital from January 2006 to December 2008. The characteristics of all patients were listed in Table 1. All 67 specimens underwent microscopic confirmation of diagnosis, histological type, and tumor grade, by two experienced pathologists (RF Cheng and Y Pan). Among 67 patients, 16 cases were not received pelvic or Para-aortic and pelvic lymphadenectomy owing to these patients with residual tumors ≥1cm. Tumor specimens and ascites were collected from EOC patients during primary surgery and prior to the initiation of adjuvant therapy. After being centrifuged at 1000 rpm for 15 min, 10 milliliters ascites were preserved at -80°C until ELISA assay. Adjuvant chemotherapy consisted of paclitaxel (175 mg/m^2) and carboplatin (6 AUC). All patients were followed until death or the end of the follow-up period (October 31, 2012). The patients' response to chemotherapy was assessed according to RECIST criteria ver. 1.1 (Eisenhauer et al., 2009) and platinum sensitivity of tumors was determined as described previously (Eisenhauer et al., 2008). Overall survival (OS) was defined as the time interval from the date of primary surgery to the date of death (failure) or to the end of follow-up for women who were alive (censored). Progression-free survival (PFS) was defined as the time elapsed from the date of primary surgery to the appearance of disease recurrence or progression (failure) or the last follow-up for women who were alive with no

Table 1. Patients' Characteristics

Characteristics	
Age at surgery (years)	Media: 51; Range: 28-76
≤51	31 (46.3%)
>51	36 (53.7%)
Menopausal status	
Yes	45 (67.2%)
No	22 (32.8%)
Pathologic type	
Serous	36 (53.7%)
Mucous	19 (28.4%)
Endometrioid	12 (17.9%)
Histologic grade	
G1-2	27 (40.3%)
G3	40 (59.7%)
FIGO Stage*	
I-II	24 (35.8%)
III-IV	43 (64.2%)
With Lymphadnectomy	51 (76.1%)
Pelvic	42 (82.4%)
Para-aortic+pelvic	9 (17.6%)
Removed Lymph nodes	Media: 26; Range: 11-56
Lymph nodes metastasis	
No	34 (66.7%)
Yes	17 (33.3%)
Residual tumor	
<1cm	51 (76.1)
≥ 1cm	16 (23.9)
Ascites volume (ml)	Media: 1000; Range: 200-7000
≤1000	22
>1000	45
Serum CA125 (U/ml)	Media: 675; Range: 23-5400
≤675	43 (64.2%)
>675	24 (35.8%)
Patients' response to chemotherapy	
CR	49 (73.1%)
PR, SD and PD	18 (26.9%)
Tumors' sensitivity to chemotherapy	
Platinum sensitive	53 (79.1%)
Platinum resistant and refractory	14 (20.9%)

*FIGO, Federation International of Gynecology and Obstetrics

evidence of disease recurrence or progression (censored).

Cell lines and culture

Ovarian cancer cell lines SKOV3 and SW626 were bought from ATCC (Rockville, MD) and A2780 was bought from Chinese Academy of Medical Sciences (Beijing). SKOV3 cells were cultured in McCoy's 5A (Gibco, USA) with 10% FBS (Invitrogen, USA); SW626 cells were cultured in Leibovitz's L-15 medium (ATCC) with 10% FBS; A2780 cells were cultured in RPMI 1640 medium (Invitrogen, USA) with 10% FBS. Human primary cultured ovarian cells (PriCells, wuhan, China), cultured in DMEM medium (ATCC) with 10% FBS, were gifted by Prof. Ren (Key Laboratory of Cancer Prevention and Therapy, Tianjin Medical University). Cells were washed twice with PBS when they grew to 80% confluence and were then kept in serum-free culture medium for an additional 48 h. Supernatant for ELISA assay was collected and debris was removed by centrifugation and then filtration through a 0.22-lm filter. Macrophages from EOC ascites and Monocytes (MOs) isolation and culture

Macrophages were isolated from ascites of EOC patients (n=67). The cells were isolated by standard Ficoll-Paque (Shanghai Hengxin Chemical Reagents Company, China) density-gradient centrifugation (2000 rpm, 10 min, 4 °C, without brake). CD14+ macrophages were purified by positive selection using magnetic-activated cell sorting (MACS) technology (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (MOs) were obtained from the peripheral blood of healthy women donors (n=10). The blood was diluted (1:2) with Hanks' balanced salt solution (Gibco, Grand Island, NY, USA), and leukocytes were fractionated by Ficoll-Hypaque gradient centrifugation (2000 rpm, 20 min, 20 °C, without brake). Then, MOs were obtained from the mononuclear cell layer according to the method of Denholm (Denholm et al., 1989). The isolated MOs were incubated with/without human recombined soluble SEMA4D protein (R&D Systems, Minneapolis, MN). All cells were cultured in 5% CO_2-humidified atmosphere at 37°C.

Immunohistochemistry and evaluation

The 4-mm thick sections were deparaffinized, rehydrated and autoclave-treated at 121°C for 10 min in 0.1 M citrate buffer (pH 6.0) to induce antigen retrieval. Endogenous peroxides in the section were blocked by incubation in 3% hydrogen peroxide for 5 min. Then, after being blocked with 0.5% goat serum for 60 min, the sections were incubated at 4°C overnight with primary antibodies CD163 (ABcam Inc. USA) or SEMA4D (BD Biosciences, USA). The DAKO REAL EnVision Detection kit (DAKO) was subsequently applied for 30 min. Finally, sections were incubated in 3'3-diaminobenzidine for 5 min, followed by Mayer's hematoxylin counterstaining and mounting. Negative controls were obtained by replacing the primary antibody with isotype-matched monoclonal antibody. The percentage of positive cells was rated on the following point scale: no points (negative), ≤10% positive cells, regardless of staining intensity; 1 point, 11%–20% positive cells; 2 points, 21%–50% positive cells; 3 points, 51%–80% positive cells; and 4 points, ≥81% positive cells. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for the percentage of positive cells and staining intensity were added, and specimens were attributed to two groups according to their overall score. Finally, specimens of ≤3 points were rated as negative, or as positive.

CD163 already had been regarded as the specific marker for M2 macrophage. The number of CD163+ macrophages was initially determined using a low-power magnification (100×). And then, CD163+ macrophages count was estimated on high-power (400×) magnification from 10 different representative fields per case (where the staining was the strongest). Only CD163+ cells displaying macrophage morphology were counted. The average counts were recorded as M2 macrophages count for each patient. Two independent investigators (RF Cheng and Y Pan) blinded to Immunohistochemistry and evaluation.
Determination of SEMA4D concentration in ascites and supernatant of cells

Determination of SEMA4D concentrations in ascites and supernatant of ovarian cancer and primary ovarian cells were performed using ELISA kits (R&D Systems, Minneapolis, MN). Each sample (100 μl) was used for the following examination. The detection thresholds were 0.1 pg/ml. The intra-assay variability was 5-20%. All tests were run in triplicate according to the manufacturer's instructions.

Cell RNA Isolation and Reversed Transcript PCR (RT-PCR) Analysis

SKOV3, SW626, A2780, and primary ovarian cell RNAs were extracted from whole cell lysates and converted into cDNA using the AMV reverse-transcriptional system (Promega) in the presence of random hexamers (Invitrogen), seperately. Then, 500 ng of cDNA was used for PCR amplification. PCR reaction products were run on 1.5% agarose gels and visualized using ethidium bromide. The gene specific primers for RT-PCR were designed using the Primer3 Input 0.4.0 software. The amplicon specificity was verified by gel running and/or by sequencing. The primers used were as followed, SEMA4D; 5'GTCTTCAAAGAAGGGCAACAGG and 5'GAGCATTTCAGTTCCGCTGTG; β-actin was as control; β-actin; 5'CCTGGGCATGGAGTCCTGTG and 5'AGGGGCCGGACTCGTCATAC.

Cell extracts and Western bolt analysis

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris-Hcl [Ph 7.4], 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin) on ice, then quantified with the Lowry method. Samples (50 μg) of the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in TBST buffer (10 mM Tris-Hcl [Ph 7.4], 150 mM NaCl, 0.1% Tween 20) at room temperature for 2 h and incubated at 4°C overnight

Table 2. Correlation Between Clinicopathologic Characteristics and SEMA4D and M2-macrophages in Tumor and Ascites in EOC Patients, Respectively

Clinicopathologic characteristic	Cases N	Positive SEMA4D in primary tumor N (%)	*P*	M2-macrophages count in primary tumor (Mean±SD)	*P*	SEMA4D in Ascites (Mean ± SD, pg/ml)	*P*	M2-macrophages in Ascites (Mean±SD%)	*P*
Age (years)			0.626		0.971		0.36		0.124
≤50	31	18 (58.1)		80±17		428±112		62.9±9.6	
>50	36	23 (63.9)		80±18		489±81		66.5±9.2	
Menopause			0.805		0.686		0.874		0.693
Yes	45	28 (62.2)		80±17		465±123		64.5±10.0	
No	22	13 (59.1)		79±18		454±87		65.5±8.4	
Pathologic type			0.274		0.613		0.979		0.822
Serous	36	23(63.9)		79±20		456±55		64.2±8.9	
Mucous	19	9 (47.4)		80±14		472±147		65.4±11.8	
Endometrioid	12	9(75.0)		84±12		460±140		65.9±7.7	
Histologic grade			0.001		0.016		0.02		0.133
G1-2	27	10 (37.0)		73±20		374±116		62.7±10.7	
G3	40	31 (77.5)		85±13		520±88		66.3±8.5	
FIGO Stage			0.003		0.038		0.084		<0.001
I-II	24	9 (37.5)		73±22		385±114		57.8±9.3	
III-IV	43	32 (74.4)		84±12		504±90		68.8±7.2	
LN metastasis			0.003		0.167		0.226		0.001
No	34	15 (44.1)		78±20		367±97		60.9±10.7	
Yes	17	15 (88.2)		84±9		449±142		69.2±5.7	
Residual tumor			0.194		0.436		0.001		0.153
<1cm	51	29 (56.9)		79±17		399±115		63.9±9.9	
≥1cm	16	12 (80.0)		83±20		659±111		67.8±7.7	
Ascites volume (ml)			0.435		0.839		0.003		<0.001
≤1000	22	12(54.5)		81±19		343±74		54.5±6.6	
>1000	45	29 (64.4)		80±17		519±90		69.9±6.0	
Serum CA125 (U/ml)			0.226		0.829		0.725		0.816
≤675	43	24 (55.8)		80±18		452±80		64.7±10.6	
>675	24	17 (70.8)		79±15		477±55		65.2±7.4	
Patients' response to chemotherapy			0.005		0.568		0.063		0.005
CR	49	25 (51.0)		79±20		424±92		63.3±10.3	
PR, SD and PD	18	16 (88.9)		82±9		562±44		69.0±5.3	
Tumors' sensitivity to chemotherapy			0.034		0.018		0.003		<0.001
Platinum sensitive	53	29		83±18		411±85		56.5±10.2	
Platinum resistant and refractory	14	12		71±13		650±94		69.8±3.3	

with the indicated primary antibodies (anti-SEMA4D 1:100 dilution, BD Biosciences, San Jose, CA and β-actin 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). After the membranes had been washed with TBST buffer, they were reacted with the appropriate horseradish-peroxidase-conjgated secondary antibody for 60 min at room temperature. After the membranes had been extensively washed with TBST buffer, the proteins were visualized with enhanced chemiluminescence reagent (SuperSignal West Pico Chemilunescent Substrate; Pierce, Rockford, IL, USA).

Fluorescence-activated cell sorting (FACS) analysis

Phenotypes of MOs stimulated with/without human recombined soluble SEMA4D protein for 24 h and macrophages from ascites of EOC patients purified by CD14+ selection were analyzed by FACS (Becton Dickinson, Mountain View, Calif), using PE-labeled anti-CD14 and FITC-labeled anti-CD163 antibodies (BD Biosciences, San Jose, CA). 1×10^5 cells were incubated with antibodies for 30 min on ice in dark. Then, the cells were washed twice with PBS containing 0.2% BSA before fixing using 1% paraformaldehyde and analyzed. Results are expressed as percentages of subpopulation of immunocytes with positive markers. The isotype-matched IgG1 was used as negative control to eliminate nonspecific staining.

Statistical analysis

The t test or one-way ANOVA was used in analysis of continuous variable, while the chi-squared and Fisher's exact tests were applied in analysis of categorical variable. Spearman rank correlation was used to assess correlation among variables. Factors that were deemed of potential importance by univariate analysis were included in the multivariate analysis. $P<0.05$ was considered significantly. The 67 EOC patients were divided into low and high subgroups according to the means of CA125, SEMA4D concentration, and M2 macrophages count as cutoffs. All statistical analysis was performed with SPSS17.0 (SPSS, Chicago, IL).

Ethics Statement

All human specimens, including ascites, peripheral blood, and tumor samples, were obtained with the written informed consent of participants in accordance with the requirements of the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital, China.

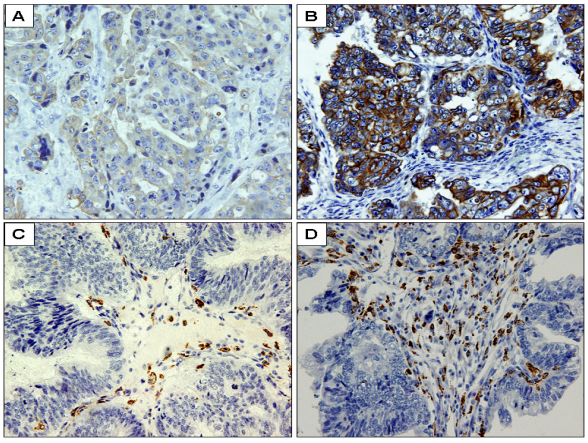

Figure 1. Representative Images Showing SEMA4D (A, B) and CD163 (C, D) Expressions Detected by IHC

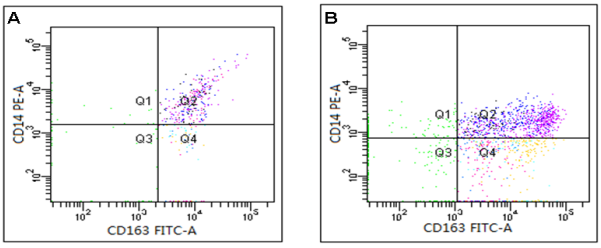

Figure 2. Representative Images Showing the Percent of M2 Macrophage in EOC Ascites Detected by FACS

Table 3. Correlation of SEMA4D and M2-macrophage in Primary Tumor and Ascites of EOC, Respectively

		M2-macrophages in primary tumor N (%)		*r*	*P*
	Cases (N)	Low	High		
SEMA4D in primary tumor				0.281	0.021
Negative	26	17 (65.4)	9 (34.6)		
Positive	41	15 (36.6)	26 (63.4)		
		M2-macrophage in ascites N(%)		*r*	*P*
		Low	High		
SEMA4D in ascites				0.355	0.003
Low	52	29 (55.8)	23 (44.2)		
High	15	2 (13.3)	13 (86.7)		

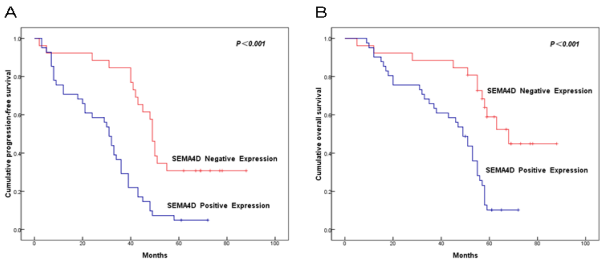

Figure 3. Kaplan–Meier Analysis for the Progression-free Survival (PFS) and Overall Survival (OS) of Epithelial Ovarian Cancer (EOC)

Results

SEMA4D expression, M2 macrophage count in primary tumors and SEMA4D expression, M2 macrophage percent in ascites and clinicopathological characteristics in EOC patients

As shown in Table 2, firstly, over-expressions of SEMA4D in primary tumor was closely related to EOC tissues with low differentiated, advanced stage, lymph node (LN) metastasis, patients' worse response to chemotherapy and platinum resistant and refractory (P<0.05, Figure 1A and 1B). Secondly, high M2 macrophages count in primary tumor was closely associated with EOC tissues with low differentiated, advanced stage and platinum resistant and refractory (P<0.05, Figure 1C and 1D). Thirdly, high SEMA4D level in ascites was closely to EOC patients with low differentiated, residual tumor ≥ 1 cm, ascites volume >1000 ml and platinum resistant and refractory (P<0.05). Moreover, high M2 macrophage percent in ascites was related to EOC patients with advanced stage, LN metastasis, ascites volume >1000 ml, patients' worse response to chemotherapy and platinum resistant and refractory (P<0.05, Figure 2). Overall, these results suggest that both over-expressions SEMA4D and M2 macrophages in primary tumor and ascites are associated with a more malignant ovarian cancer phenotype.

Correlations of SEMA4D expressions and M2 macrophage count in primary tumors and SEMA4D expression and M2 macrophage percent in ascites in EOC patients, respectively

The 67 EOC patients were categorized into high and low subgroups according to mean of M2 macrophage count in primary tumors (80), SEMA4D expression in ascites (461 pg/ml), and M2 macrophage percent in ascites (65%), respectively. In primary tumors, 41 SEMA4D positive expressions tissues, 63.4% (26/41) showed high M2 macrophage count (r=0.281, P=0.021, Table 3). Likewise, 15 high SEMA4D expression in ascites, 86.7% (13/15) showed high M2 macrophage percent (r=0.355, P=0.003, Table 3). The data showed the correlations of SEMA4D expressions and M2 macrophages count in primary tumors and SEMA4D expression and M2 macrophage percent in ascites were closely, respectively.

Survival analysis of prognosis factors in EOC

By univariate analysis, the shorter media of OS and PFS were related to low differentiated, advanced stage, LN metastasis, ascites volume >1000 ml, patients' worse response to chemotherapy, platinum resistant and refractory, over-expression of SEMA4D and high M2 macrophage count in primary tumor, and high SEMA4D level and M2 macrophage percent in ascites (P<0.05, Table 4). Furthermore, shorter media of PFS was related to residual tumor≥1cm (P=0.024, Table 4).

These significant variables detected by univariate analysis were included in multivariate analysis. In Cox proportional hazard model, histologic grade, LN metastasis or not, tumors' sensitivity to chemotherapy and SEMA4D expression in primary tumor were the independent factors for evaluation of PFS (P < 0.05, Table 4). Additionally, stage, patients' response to chemotherapy and SEMA4D expression in primary tumor were the independent factors for evaluation of OS (P < 0.05, Table 4). Thus, EOC patients with positive SEMA4D expression in primary tumor showed shorter PFS and OS than those with negative expression (Figure 3).

Table 4. Univariate and Multivariate Survival Analyses of the Prognostic Factors for Overall and Disease-free Survival in EOC Patients

Variable	Cases (N)		Progression-free survival				Overall survival				
			Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis		
			Media of PFS	P^a	HR	95% CI For HR	P^b	Media of OS	P^a	HR	95% CI For HR	P^b
Age (years)				0.321	NA	NA	NA		0.14	NA	NA	NA
≤51	31	39						58				
>51	36	32						50				
Menopause				0.856	NA	NA	NA		0.831	NA	NA	NA
Yes	45	36						55				
No	22	40						55				
Pathologic type				0.237	NA	NA	NA		0.403	NA	NA	NA
Serous	36	40						55				
Mucous	19	36						58				
Endometrioid	12	31						51				
Histologic grade				<0.001	2.843	1.212-6.665	0.016		<0.001	2.403	0.945-6.110	0.066
G1-2	27	49						59				
G3	40	30						48				
FIGO Stage				<0.001	1.452	0.457-4.620	0.527		<0.001	0.509	0.289-0.894	0.019
I-II	24	49						63				
III-IV	43	30						48				
LN metastasis				<0.001	0.507	0.299-0.859	0.012		<0.001	4.074	0.879-18.877	0.073
No	34	48						63				
Yes	17	18						35				
Residual tumor				0.024	0.542	0.061-4.796	0.582		0.177	NA	NA	NA
<1cm	51	40						55				
≥1cm	16	31						43				
Ascites volume (ml)				<0.001	1.061	0.314-3.579	0.924		<0.001	0.649	0.158-2.674	0.55
≤1000	22	49						58				
>1000	45	31						49				
Serum CA125 (U/ml)				0.753	NA	NA	NA		0.991	NA	NA	NA
≤675	43	36						55				
>675	24	39						55				
Patients' response to chemotherapy				<0.001	0.913	0.237-3.527	0.895		<0.001	0.243	0.065-0.906	0.035
CR	49	43						58				
PR, SD and PD	18	9						20				
Tumors' sensitivity to chemotherapy				<0.001	21.272	3.434-131.759	0.001		<0.001	0.493	0.241-1.009	0.053
Platinum sensitive	53	43						58				
Platinum resistant and refractory	14	7						15				
SEMA4D in tumor				<0.001	0.509	0.289-0.869	0.021		<0.001	4.394	1.701-11.350	0.002
Negative	26	49						68				
Positive	41	31						49				
M2-macrophage count in tumor				0.025	2.151	0.894-5.175	0.087		0.006	1.608	0.599-4.312	0.346
≤80	32	42						58				
>80	35	34						53				
SEMA4D in ascites (pg/ml)				0.001	1.136	0.336-3.841	0.837		0.002	2.591	0.750-8.954	0.132
≤461	52	40						57				
>461	15	20						32				
M2-macrophages percent in ascites (%)				<0.001	2.047	0.694-6.037	0.194		0.001	1.852	0.591-5.798	0.29
≤65	31	31						58				
>65	36	36						47				

P^a, P value, log rank test; HR, hazard ratio; CI, confidence interval; P^b, P value, Cox regression; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NA , not applicable

Higher SEMA4D was secreted and expressed by ovarian cancer cell and SEMA4D switches MOs phenotype into TAM-like cells

The results demonstrate that SEMA4D in supernatant secreted by ovarian cancer cell SKOV3, SW626 and A2780 were obviously higher than human primary cultured ovarian cells (P<0.001, Figure 4A). Additionally, higher expressions of SEMA4D in SKOV3, SW626 and A2780 were detected by RT-PCR and Western blot than in human primary cultured ovarian cells, respectively (Figure 4B and C). Moreover, human recombined soluble SEMA4D protein treated MOs demonstrated a higher percentage of cells expressing CD163 compared to untreated MOs (27.7±2.8% vs 16.8±2.7%, P=0.014, Figure 4D-b), although there were not significantly different percentage of cells expressing CD14 in untreated MOs, MOs treated with SEMA4D and macrophages from EOC ascites (83.6±4.0 vs 84.3±2.6 vs 86.5±6.3, P>0.05,

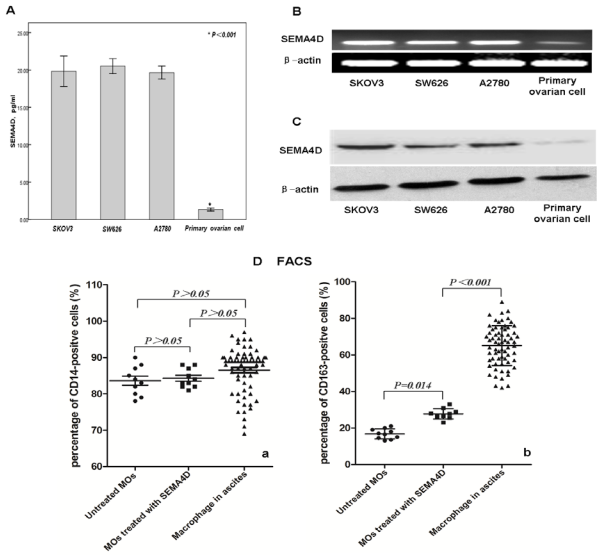

Figure 4. A, B and C: SEMA4D expressions in ovarian cancer cells and human primary ovarian cell were investigated by ELISA, RT-PCR and Western blot, respectively; D: Phenotypes of Monocytes (MOs, n=10) with/without SEMA4D soluble protein simulation and macrophage from EOC ascties (n=67) were detected by FACS

Figure 4D-a). Additionally, even though the percentage of cells positive for CD163 in MOs treated with SEMA4D was lower than that of macrophages from ascites (27.7±2.8% vs 65.1±10.9%, $P<0.001$, Figure 4D-b), there was a shift toward a M2 macrophage-like phenotype.

Discussion

Although semaphorins were originally identified as molecules regulating a functional activity of axons in the nervous system, SEMA4D recently have been implicated in a host of responses including regulation of cell migration, angiogenesis, immune and inflammation responses, and tumor progression. Emerging evidences identified SEMA4D was highly expressed in some prevalent solid tumors, including breast, prostate, colon carcinomas, and HNSCC (Basile et al., 2006). However, expression of SEMA4D in EOC microenvironment was not well understood. Interestingly, SEMA4D, a membrane bound protein, must be processed and released into a soluble form to act in tumor surrounding microenvironment to regulate angiogenesis and tumor progression (Basile et al., 2007). Accordingly, in this study, we showed that over-expression of SEMA4D in primary tumor or ascites was closely related to EOC patients with a more malignant phenotype, which also firstly clarified the expression of SEMA4D in EOC ascites.

Macrophages have two different functions, a tumor-suppressive (M1) function and a tumor-supportive (M2) function. TAMs display the M2 macrophage phenotype and are well-studied for their role in creating a permissive microenvironment for tumor growth. CD163, more specific than CD68 marker, is expressed in the M2 macrophages. Several studies had already proved that high M2 macrophage count indicated tumor more aggressive and poor prognosis, including breast cancer (Shabo et al., 2008), lung adenocarcinoma (Zhang et al., 2011), and classical Hodgkin's lymphoma (Sanchez-Espiridion et al., 2012). Moreover, Lan et al. recently showed that the infiltration of CD163-positive M2 macrophages as well as activation of macrophages towards the M2 phenotype may contribute to poor survival in advanced ovarian cancer (Lan et al., 2013), which was consistent with our results that demonstrated that both high M2 macrophage count in primary tumor and percent in ascites were closely related to EOC patients with advanced stage and platinum resistant and refractory and suggested a shorter OS and PFS.

Furthermore, researchers almost had already reached the consensus that angiogenesis is prominent in sites of tumor invasion and metastasis. Indeed, M2 macrophages are a major player in the regulation of tumor angiogenesis. Shabo I et al. reported expression of the M2 macrophage antigen CD163 in rectal cancer cells is associated with early local recurrence and reduced survival time (Shabo et al., 2009). However, SEMA4D, recently, as a newfound pro-angiogenic player during tumor angiogenesis, caused more and more attention. Studies proved SEMA4D regulate blood vessel growth and endothelial cell homing during vessel development (Carmeliet et al., 2005). Zhou et al. examined the contribution of SEMA4D to tumor-induced angiogenesis when compared to VEGF and suggested that targeting these proteins might represent a complementary or parallel mode of treatment for anti-angiogenic therapy of HNSCC and other solid tumors exhibiting insensitivity of anti-VEGF therapy (Zhou et al., 2012). Notably, our previous study demonstrated over-expression of SEMA4D was positively correlated with EOC angiogenesis (Chen et al., 2012). Complementally, in this study, the results also identified the correlations of SEMA4D and M2 macrophages count in primary tumors or SEMA4D and M2 macrophage percent in ascites were closely, respectively, which implied the SEMA4D associated with M2 macrophage maybe play coincident roles in promoting tumor angiogenesis and the herein mechanism deserved investigated further.

In addition, the univariated and multivariate survival analysis showed SEMA4D over-expression was the independent factor for predicting OS and PFS of EOC patients, which was similarly with recent work that a correlation between high levels of SEMA4D expression in some sarcomas and poor overall patient prognosis (Ch'Ng et al., 2007).

Besides, in this study, higher expressions of SEMA4D in ovarian cancer cell lines (SKOV3, A2780, and SW626) and their supernatants were observed than that in human primary cultured ovarian cell and its supernatant, which verified the phenomena that we showed before in EOC and normal ovarian tissues (Chen et al., 2012) and provided the appropriated cell model in vitro for further investigation. Complementally, we determined that human soluble SEMA4D protein could mediate polarization of MOs toward M2-like phenotypes with characteristics of increasing CD163 expression, which was analogous to other literatures that confirmed several cytokines, associated with immune and inflammation response, alters the phenotype of tumor-associated macrophages from M2 to M1, including coagulation factor XII (FXII),

COX-2 inhibition, IL-10 (Sica et al., 2008; Wang et al., 2010; Nakanishi et al., 2011). Further validated work that SEM4D, as important player in immune and inflammation, induced macrophage polarization in tumor progression is needed in the future.

In summary, we found SEMA4D expression was positively correlated with M2 macrophage in EOC and was a promising indicator of poor prognosis for EOC patients. Furthermore, our data indicates that human recombined soluble SEMA4D protein may act as a novel mediator stimulating MOs towards the M2 macrophage phenotype in vitro. This newfound relationship between SEMA4D and M2 macrophage in EOC may help understand the role of SEMA4D in promoting EOC dissection and metastasis in tumor microenviroment, and expect that SEMA4D may serve as a reliable tool for early and accurate prediction of tumor recurrence and may be a potential therapeutic target for EOC patients.

Acknowledgements

This work was supported by Tianjin Health Bureau of Science and Technology Funds (2012KZ073) and National Natural Science Foundation (81302250). The author(s) declare that they have no competing interests.

References

Abdollahi T, Robertson NM, Abdollahi A, et al (2003). Identification of interleukin 8 as an inhibitor of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in the ovarian carcinoma cell line OVCAR3. *Cancer Res*, **63**, 4521-6.

Auersperg N, Ota T, Mitchell GW (2002). Early events in ovarian epithelial carcinogenesis: progress and problems in experimental approaches. *Int J Gynecol Cancer*, **12**, 691-703.

Basile JR, Castilho RM, Williams VP, et al (2006). Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis. *Proc Natl Acad Sci U S A*, **103**, 9017-22.

Basile JR, Holmbeck K, Bugge TH, et al (2007). MT1-MMP controls tumor-induced angiogenesis through the release of semaphorin 4D. *J Biol Chem*, **282**, 6899-905.

Bast RJ, Hennessy B, Mills GB (2009). The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer*, **9**, 415-28.

Carmeliet P, Tessier-Lavigne M (2005). Common mechanisms of nerve and blood vessel wiring. *Nature*, **436**, 193-200.

Chen Y, Zhang L, Pan Y, et al (2012). Over-expression of semaphorin4D, hypoxia-inducible factor-1α and vascular endothelial growth factor is related to poor prognosis in ovarian epithelial cancer. *Int J Mol Sci*, **13**, 13264-74.

Ch'Ng E, Tomita Y, Zhang B, et al (2007). Prognostic significance of CD100 expression in soft tissue sarcoma. *Cancer*, **110**, 164-72.

Delaire S, Billard C, Tordjman R, et al (2001). Biological activity of soluble CD100. II. Soluble CD100, similarly to H-SemaIII, inhibits immune cell migration. *J Immunol*, **166**, 4348-54.

Denholm EM, Wolber FM, Phan SH (1989). Secretion of monocyte chemotactic activity by alveolar macrophages. *Am J Pathol*, **135**, 571-80.

Eisenhauer EA, Therasse P, Bogaerts J, et al (2009). New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer*, **45**, 228-47.

Eisenhauer EL, Abu-Rustum NR, Sonoda Y, et al (2008). The effect of maximal surgical cytoreduction on sensitivity to platinum-taxane chemotherapy and subsequent survival in patients with advanced ovarian cancer. *Gynecol Oncol*, **108**, 276-81.

Giuntoli RN, Webb TJ, Zoso A, et al (2009). Ovarian cancer-associated ascites demonstrates altered immune environment: implications for antitumor immunity. *Anticancer Res*, **29**, 2875-84.

Ishida I, Kumanogoh A, Suzuki K, et al (2003). Involvement of CD100, a lymphocyte semaphorin, in the activation of the human immune system via CD72: implications for the regulation of immune and inflammatory responses. *Int Immunol*, **15**, 1027-34.

Jensen TO, Schmidt H, Moller HJ, et al (2009). Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *J Clin Oncol*, **27**, 3330-7.

Kumanogoh A, Kikutani H (2004). Biological functions and signaling of a transmembrane semaphorin, CD100/Sema4D. *Cell Mol Life Sci*, **61**, 292-300.

Lan C, Huang X, Lin S, et al (2013). Expression of M2-polarized macrophages is associated with poor prognosis for advanced epithelial ovarian cancer. *Technol Cancer Res Treat*, **12**, 259-67.

Lane D, Matte I, Rancourt C, et al (2011). Prognostic significance of IL-6 and IL-8 ascites levels in ovarian cancer patients. *BMC Cancer*, **11**, 210.

Lee CH, Espinosa I, Vrijaldenhoven S, et al (2008). Prognostic significance of macrophage infiltration in leiomyosarcomas. *Clin Cancer Res*, **14**, 1423-30.

Martinez FO, Sica A, Mantovani A, et al (2008). Locati M. Macrophage activation and polarization. *Front Biosci*, **13**, 453-61.

Nakanishi Y, Nakatsuji M, Seno H, et al (2011). COX-2 inhibition alters the phenotype of tumor-associated macrophages from M2 to M1 in ApcMin/+ mouse polyps. *Carcinogenesis*, **32**, 1333-9.

Qian BZ, Pollard JW (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell*, **141**, 39-51.

Sakurai A, Doci CL, Gutkind JS (2012). Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. *Cell Res*, **22**, 23-32.

Sanchez-Espiridion B, Martin-Moreno AM, Montalban C, et al (2012) F. Immunohistochemical markers for tumor associated macrophages and survival in advanced classical Hodgkin's lymphoma. *Haematologica*, **97**, 1080-4.

Shabo I, Olsson H, Sun XF, et al (2009). Expression of the macrophage antigen CD163 in rectal cancer cells is associated with early local recurrence and reduced survival time. *Int J Cancer*, **125**, 1826-31.

Shabo I, Stal O, Olsson H, et al (2008). Breast cancer expression of CD163, a macrophage scavenger receptor, is related to early distant recurrence and reduced patient survival. *Int J Cancer*, **123**, 780-6.

Sica A, Larghi P, Mancino A, et al (2008). Macrophage polarization in tumour progression. *Semin Cancer Biol*, **18**, 349-55.

Siegel R, Naishadham D, Jemal A (2012). Cancer statistics. *CA Cancer J Clin*, **62**, 10-29.

Sierra JR, Corso S, Caione L, et al (2008). Tumor angiogenesis and progression are enhanced by Sema4D produced by tumor-associated macrophages. *J Exp Med*, **205**, 1673-85.

van Dongen M, Savage ND, Jordanova ES, et al (2010). Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. *Int J Cancer,* **127**, 899-909.

Wang R, Zhang T, Ma Z, et al (2010). The interaction of coagulation factor XII and monocyte/macrophages mediating peritoneal metastasis of epithelial ovarian cancer. *Gynecol Oncol*, **117**, 460-6.

Zhang B, Yao G, Zhang Y, et al (2011). M2-polarized tumor-associated macrophages are associated with poor prognoses resulting from accelerated lymphangiogenesis in lung adenocarcinoma. *Clinics (Sao Paulo)*, **66**, 1879-86.

Zhang T, Ma Z, Wang R, et al (2010). Thrombin facilitates invasion of ovarian cancer along peritoneum by inducing monocyte differentiation toward tumor-associated macrophage-like cells. *Cancer Immunol Immunother*, **59**, 1097-108.

Zhou H, Binmadi NO, Yang YH, et al (2012). Semaphorin 4D cooperates with VEGF to promote angiogenesis and tumor progression. *Angiogenesis*, **15**, 391-407.