



# Axon guidance repulsant SEMA3F increases chemosensitivity to oxaliplatin and inhibits epithelial-mesenchymal transition of colorectal cancer cells

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**Background:** Our previous study has shown that down-regulation of axon guidance repulsive Semaphorin-3F (SEMA3F) promotes growth and metastasis of colorectal cancer (CRC) cells. However, the role of SEMA3F in chemosensitivity and epithelial-mesenchymal transition of CRC cells remains unknown.

**Methods:** The expression of SEMA3F, P-gp, GST- $\pi$  and TOPO-II in CRC tissues from 94 patients was determined by immunohistochemical staining. Knock-down of SEMA3F was achieved by lentivirus transfection. Functional assays were done to evaluate the invasion, apoptosis and cell viability.

**Results:** We found that the expression of SEMA3F is negatively correlated with the expression of P-gp and GST- $\pi$  in CRC tissues from 94 patients. Knock-down of SEMA3F significantly decreased apoptosis of HCT116 and SW480 cells, accompanying with up-regulation of anti-apoptotic BCL-2 and down-regulation of pro-apoptotic BAD and BAX. In addition, knock-down of SEMA3F attenuated chemosensitivity to oxaliplatin of CRC cells. Moreover, we found that down-regulation of SEMA3F promotes epithelial-mesenchymal transition of HCT116 and SW480 cells by decreasing the expression of epithelial marker E-cadherin, and increasing the expression of mesenchymal marker Slug, Snail and Vimentin. Finally, knock-down of SEMA3F increased the tumor volume and decreased overall survival of nude mice using colorectal cancer orthotopic xenograft model under the treatment of oxaliplatin.

**Conclusions:** SEMA3F increases chemosensitivity to oxaliplatin and inhibits epithelial-mesenchymal transition of CRC cells.

**Keywords:** Semaphorin-3F (SEMA3F); oxaliplatin; chemosensitivity; epithelial-mesenchymal transition (EMT); apoptosis

Submitted Aug 09, 2016. Accepted for publication Nov 15, 2016.

doi: 10.21037/tcr.2017.01.15

View this article at: <http://dx.doi.org/10.21037/tcr.2017.01.15>

## Introduction

Axon guidance repulsive Semaphorin-3F (SEMA3F) belongs to the class 3 Semaphorin family, which is a large group of secreted axon guidance molecules involved in

the development of nervous system (1). Recent studies have revealed that SEMA3F was widely expressed outside the nervous system and played major roles in the tumor context such as angiogenesis, tumor growth and metastasis (2,3). The SEMA3F gene locates in 3p21.3, a region that is

frequently lost in lung cancer and breast cancer, implying that the SEMA3F gene a candidate tumor suppressor gene (4,5). In addition to gene deletion, SEMA3F has been shown to be down-regulated in many kinds of cancers such as lung (4,6), breast (7), ovarian (8), colorectal cancer (CRC) (9), and melanoma (10). Functionally, SEMA3F has been reported to inhibit proliferation, invasion, adhesion of cancer cells (1). However, the role of SEMA3F in chemosensitivity of cancer cells remains elusive.

CRC is the third most common cancer and the fourth leading cause of tumor associated death in the world (11). The incidence of CRC is still increasing in Asia, especially in China (12). Despite numerous efforts have been put into the development of diagnosis and treatment of CRC, the 5-year survival rate of CRC patients remains about 50%, with the five-year survival rate of patients with distant metastasis as low as 10–20% (11). Tumor metastasis is a sophisticated multi-step process, including escape from the primary tumor, intravasation into the lymphatic or vascular systems, survival in the circulation, avoidance of host defense mechanisms, arrest at a new site, extravasation into the tissue, and growth at the new site (13). Since the introduction of oxaliplatin for the treatment of metastatic CRC, improved objective response rate, increased percentage of metastasis resection and prolonged survival have been shown in 40–50% of the CRC patients (14). However, the remaining patients could not benefit from oxaliplatin because of side effects and the drug resistance. Oxaliplatin exerts its cytotoxic effect mainly by inducing DNA damage, which is followed by activation of apoptosis pathways (15). The molecular mechanisms underlying oxaliplatin resistance includes intracellular transport and detoxification, altered DNA damage repair and cell death mechanisms (15). The epithelial-mesenchymal transition (EMT) refers to the program by which epithelial cells shed their differentiated features and acquire mesenchymal characteristics such as motility, invasiveness and a heightened resistance to apoptosis (16). The EMT has been implicated in two of the most important processes responsible for cancer-related mortality: distant metastasis and therapeutic resistance. Remarkably, two recent studies have demonstrated that the EMT process attenuated chemosensitivity to chemotherapy drugs rather than metastasis of breast and pancreatic cancer (17,18).

Our previous study has demonstrated that SEMA3F is down-regulated in CRC samples and functions as an inhibitor of growth (19), metastasis (9) and stemness (20) of colorectal cancer cells, but the role of SEMA3F in EMT and

drug resistance to oxaliplatin was poorly known. The present work found that the expression of SEMA3F is negatively correlated with the expression of permeability glycoprotein (P-gp) and glutathione-s-transferase- $\pi$  (GST- $\pi$ ) in CRC tissues from 94 patients. Down-regulation of SEMA3F by lentivirus particles expressing shRNA against SEMA3F significantly decreased apoptosis and enhanced invasion capacity of CRC HCT116 and SW480 cells. Moreover, decreased epithelial marker E-cadherin expression, and increased mesenchymal marker Slug, Snail and Vimentin expression were found in SEMA3F down-regulating cells. The IC<sub>50</sub> to oxaliplatin of SEMA3F down-regulating cells was dramatically higher than that of control cells. *In vivo*, we showed that down-regulation of SEMA3F increased the tumor volume and decreased overall survival of nude mice using orthotopic CRC xenograft model, even under the treatment with oxaliplatin. Taken together, these results demonstrated that SEMA3F increases chemosensitivity to oxaliplatin and inhibits EMT of CRC cells.

## Methods

### Cell lines and patient samples

Ninety-four cases of tumor tissues were from colorectal cancer patients who underwent colectomy with lymph node dissection in the period from 2006 to 2008 at Southwest Hospital of Third Military Medical University. The patients received neither chemotherapy nor radiotherapy before surgery. The diagnoses of colorectal carcinoma were made independently by at least 2 histopathologists. This study was carried out in according to the principles of the Helsinki Declaration and approved by the Ethical Committee of the Third Military Medical University (No. 2013062). Written informed consent was obtained from all patients.

The human CRC cell line HCT116, SW480, SW620, HT29 and LoVo were obtained from the American Type Culture Collection. The ATCC catalogs of the cells used in the present study are as follows: SW480, ATCC<sup>®</sup> CCL-228<sup>TM</sup>; SW620, ATCC<sup>®</sup> CCL-227<sup>TM</sup>; HCT116, ATCC<sup>®</sup> CCL-247<sup>TM</sup>; HT29, ATCC<sup>®</sup> HTB-38<sup>TM</sup>; LoVo, ATCC<sup>®</sup> CCL-229<sup>TM</sup>. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 U/mL penicillin-streptomycin, vitamins, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and nonessential amino acids (Thermo Scientific, Watertown, MA, USA) at 37 °C in 5% CO<sub>2</sub>.

### ***Immunohistochemical staining***

Following deparaffinized and rehydrated, tumor sections of 4  $\mu\text{m}$  were incubated in 0.3%  $\text{H}_2\text{O}_2$  in methanol for 30 minutes at 37 °C to block endogenous peroxidase. The sections were then boiled in 10 mmol/L citrate buffer (pH =6.0) for 15 minutes in a microwave oven. The antibodies against SEMA3F (Millipore, Billerica, MA, USA), P-gp (MXB Biotechnologies, China), GST- $\pi$  (MXB Biotechnologies, China) or Topo-II (MXB Biotechnologies, China) was added and the sections were incubated at 4 °C overnight. The sections were visualized using the diaminobenzidine solution (DAKO), and then lightly counterstained with hematoxylin. Sections without incubation with primary antibody served as negative controls. The intensity of staining (brown color) was semi-quantitatively scored as follows: 1, weak; 2, medium; 3, strong; and 4, very strong. The percentage of maximally stained tumor cells in each section was recorded (0, <5%; 1, 5–30%; 2, 30–50%; 3, >50%). High expression of SEMA3F was defined as a combined score for the intensity and area of staining that was  $\geq 2$ . The results were verified by two histopathologists independently.

### ***Knock-down of SEMA3F***

Lentivirus particles expressing short hairpin RNA (shRNA) against SEMA3F, or control shRNA were produced by Neuronbiotech (Shanghai, China) using the sequence we previously used (19). Stable transfectant cells expressing enhanced GFP were isolated with a flow cytometric sorting system (BD FACS Aria II, Bedford, MA, USA). HCT116 cells transfected with shRNA-SEMA3F were named Lv-shSEMA3F, and the cells transfected with scramble sequence were named Lv-control, cells without transfection were named WT.

### ***Quantitative reverse transcriptase-PCR (qRT-PCR)***

Total RNA was extracted from cultured cells with Trizol Reagent (TaKaRa, Dalian, China). The concentration and integrity of extracted RNA was examined by UV spectrophotometer. The High Fidelity RNA PCR Kit and SYBR Primescript RT-PCR Kit were purchased from TaKaRa Biotech (Dalian, China). Primers for quantitative PCR were designed using Primer Premier 5.0 software according to the sequence data in the GeneBank. The primers used are as following: SEMA3F

(forward: CGAGTGTGGGAACTTCGTCA, reverse: CACATAGGTGCACATGGGGT) and GAPDH (forward: CTATAAATTGAGCCCGCAGCC, reverse: GCGCCCAATACGACCAAATC). The results of three independent experiments were analyzed using the  $\Delta\Delta\text{C}[t]$  method (21).

### ***Western blot***

Total proteins of the cultured cells were extracted with RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with inhibitors of protease and phosphatase. Proteins were then denatured and applied to electrophoresis with SDS-PAGE, followed by being transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Following incubation with 5% nonfat milk in PBST for 2 h at room temperature, the membranes were incubated with primary antibody against SEMA3F (Millipore, Billerica, MA, USA), BCL-2, BAX, BAD, p53, E-cadherin, Vimentin, Snail, Slug or GAPDH antibody (Cell Signaling, Beverly, MA, USA) overnight at 4 °C. The protein abundance was revealed by using HRP-conjugated secondary antibodies (Beyotime, Shanghai, China) and visualized with an enhanced chemiluminescence (ECL) detection system (Thermo Scientific, Watertown, MA, USA).

### ***Immunofluorescence and confocal laser scanning microscopy***

Cells transfected with lentivirus were grown on poly-L-Lysine-coated glass coverslips and then fixed for 20 minutes in 4% paraformaldehyde at room temperature. The cells were then rinsed 3 times with PBS for 5 minutes each time, followed by incubating in a protein-blocking solution for 30 minutes at 37 °C. After incubation with the primary antibody against human SEMA3F (1:100; Millipore, Billerica, MA, USA) overnight at 4 °C and being rinsed with PBS, the cells were incubated with the secondary antibody (TRITC-conjugated IgG, 1:100, Beyotime, Shanghai, China) at 37 °C for 30 minutes and then stained with DAPI (Beyotime, Shanghai, China). The slides were then analyzed by confocal laser scanning microscopy.

### ***Enzyme-linked immunosorbent assay (ELISA)***

Lentivirus transfected cells plated in 6-wells plates were cultured in fresh culture medium for 24 h. The supernatant was then collected after 24 h and the concentration of SEMA3F was measured using ELISA kits (MyBioSource,

San Diego, CA, USA) as previously described (22). The results were presented as the concentration of SEMA3F per  $10^6$  cells.

### *Apoptosis assay*

Cells seeded in 6-wells plates were incubated in fresh culture medium supplemented with or without 10  $\mu$ M oxaliplatin for 12 h. The cells were then collected with accutase (Thermo Scientific, Watertown, MA, USA) and stained with AnnexinV-APC and PI (eBioscience, San Diego, CA, USA) according to the manufacture's instructions. The cells were then analysed using a flow cytometric sorting system (BD FACS Aria II, Bedford, MA, USA).

### *Cell viability assay*

To test the proliferation of Lv-shSEMA3F and Lv-control cells, five hundred cells were seeded in 96-wells plates. The cells were then incubated with the CCK-8 (Beyotime, Shanghai, China) for 4 h at the indicated time points. Cell viability was also determined by CCK-8 kit (Beyotime, Shanghai, China). One thousand cells were seeded and cultured in 96-wells plates. After overnight recovery, the cells were incubated in fresh culture medium containing different concentration of oxaliplatin (Sigma-Aldrich, St. Louis, MO, USA) for 72 h. The half-maximal inhibitory concentration (IC50) was calculated by the Graph Pad software.

### *Cell invasion assays*

The invasion capacity of HCT116 and SW480 cells was evaluated in transwells (BD Falcon, Franklin Lakes, NJ, USA). The cells ( $5 \times 10^4$ /well) in serum-free medium were seeded into Matrigel™ (BD Biosciences, Franklin Lakes, NJ)-coated upper chambers. After incubation for 36 hours at 37 °C, the remaining cells left in the upper chamber were removed and the cells in the lower chambers were fixed and stained with crystal violet. The number of cells was counted in 5 distinct areas at  $\times 400$  magnification. The results represent the average cell number in 3 wells per cell line.

### *Orthotopic xenografts*

Four-week-old male nude mice were caged in groups of 5 and acclimated for a week. Animal care was provided according to the Guidelines for the Care and Use of Laboratory Animals. To establish orthotopic xenografts, the

mice were anesthetized with chloral hydrate and subjected to laparotomy (n=40). Afterwards,  $5 \times 10^6$  cells (Lv-shSEMA3F or Lv-control) in 20  $\mu$ L Matrigel containing growth factors (BD Biosciences, Franklin Lakes, NJ, USA) were injected into the subserosa of the mouse colon. Three weeks after implantation, mice were divided into four treatment groups (n=10 per group). Group one were injected with Lv-shSEMA3F cells and received PBS, group two were injected with Lv-shSEMA3F cells and received oxaliplatin 6 mg/kg/week i.p, group three were injected with Lv-control cells and received PBS, group four were injected with Lv-control cells and received oxaliplatin 6 mg/kg/week i.p. Xenografts were harvested and fixed in 10% formalin until the mouse died. The tumor volume was calculated using the following equation: Length  $\times$  width<sup>2</sup>  $\times$  0.52.

### *Statistical analysis*

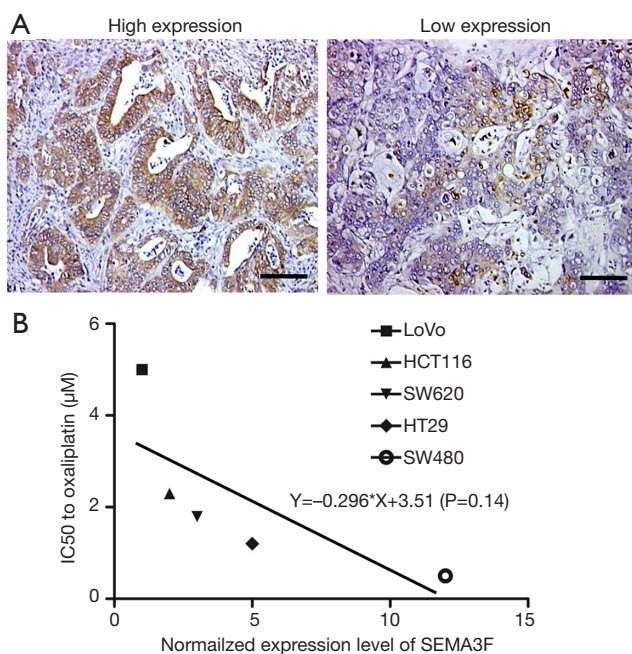
All experiments were conducted at least three times and the results were from representative experiments. Data were expressed as mean values  $\pm$  standard deviation (SD), and the statistical significance between testing and control groups was analysed with SPSS18.0 statistical software. Chi-square analysis was used to analyze the relationship between the expression of SEMA3F and drug resistance-related molecules. When two groups were compared, the unpaired Student's *t*-test was used. The Kaplan-Meier method was used to analyze overall survival of mice. P value less than 0.05 was considered statistically significant.

## **Results**

### *Negative correlation between the expression of SEMA3F and the expression of P-gp and GST- $\pi$ in CRC tissues*

We first examined SEMA3F expression in the specimens from 94 CRC patients, and found that the tumors from 53.2% (50/94) of CRC patients had low SEMA3F expression (Figure 1A). The correlation between SEMA3F expression levels and the expression of P-gp, GST- $\pi$  or topoisomerase II (Topo-II) are summarized in Table 1. We found that the expression of SEMA3F is negatively correlated with the expression of P-gp and GST- $\pi$ , but not Topo-II in CRC tissues from 94 patients. Moreover, the expression level of SEMA3F was determined by RT-PCR (Figure S1), which was correlated with the chemosensitivity to oxaliplatin of these CRC cell lines though without significant difference (Figure 1B). These results indicate that





**Figure 1** The expression of SEMA3F in CRC tissues and CRC cells. (A) Representative image of IHC staining of SEMA3F in CRC tissues; (B) the expression of SEMA3F in CRC cell line was determined using RT-PCR and normalized to the expression level in LoVo cells. The correlation between the expression of SEMA3F and the sensitivity to oxaliplatin was determined by regression analysis. Scale bar, 50 μm. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

SEMA3F is involved in the drug resistance of CRC cells.

#### *Knock-down of SEMA3F in HCT116 cells by lentivirus transfection*

The expression of SEMA3F in CRC cell lines was determined by RT-PCR, showing the expression level of SEMA3F is highest in SW480 cells and lowest in LoVo cells. (Figure S1) The expression level of SEMA3F in HCT116 cells is moderate. HCT116 and SW480 cells were transfected with lentivirus particle expressing shRNA against SEMA3F or scramble sequence. Knock-down of SEMA3F was confirmed by RT-PCR, Western blot and ELISA in SW480 cells (Figure 2A,B,C) and HCT116 cells (Figure 2D,E,F). There was no significant difference between the Lv-control cells and WT cells in the expression or secretion of SEMA3F. On the other hand, the expression or secretion of SEMA3F was significantly reduced in Lv-shSEMA3F cells. These results confirm knock-down of SEMA3F in HCT116 and SW480 cells.

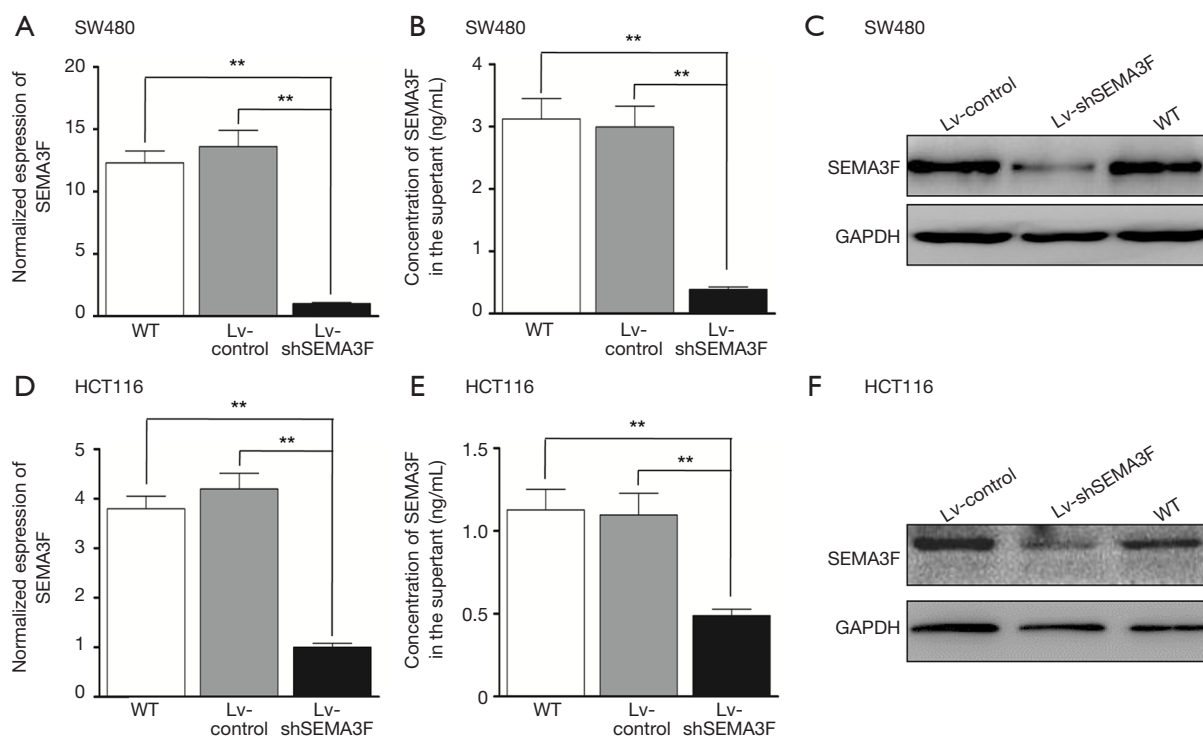
#### *Reduced apoptosis percentage of SEMA3F knock-down cells*

We first test the apoptosis percentage of Lv-shSEMA3F cells and Lv-control cells by AnnexinV-APC and PI staining. The result showed that knock-down of SEMA3F

**Table 1** Correlation between SEMA3F expression and drug resistance-related proteins in colorectal cancer tissues from 94 patients

Protein name	Expression level	the status of SEMA3F expression		P value
		Low (n=50) (%)	High (n=44) (%)	
P-gp	Positive	32 (68.1)	15 (31.9)	0.0038
	Negative	18 (38.3)	29 (61.7)	
GST	Positive	25 (65.8)	13 (34.2)	0.0438
	Negative	25 (44.6)	31 (55.4)	
Topo-II	Positive	23 (59.0)	16 (41.0)	0.3441
	Negative	27 (49.1)	28 (50.9)	

P-gp, permeability glycoprotein; GST- $\pi$ , glutathione-s-transferase- $\pi$ ; Topo-II, topoisomerase II.



**Figure 2** Knock-down of SEMA3F in SW480 and HCT116 cells by lentivirus transfection. (A) Confirmation of knock-down of SEMA3F in SW480 cells transfected with lentivirus particles containing shRNA against SEMA3F (Lv-shSEMA3F) by qRT-PCR. The expression of SEMA3F was normalized to the expression in Lv-shSEMA3F cells. There was no significant difference between the expression of SEMA3F in SW480 cells without transfection (WT) and SW480 cells transfected with lentivirus particles containing scramble sequence (Lv-control); (B) confirmation of knock-down of SEMA3F in SW480 cells by Western blot; (C) ELISA assay showed that the secretion of SEMA3F was reduced in Lv-shSEMA3F cells; (D,E,F) confirmation of knock-down of SEMA3F in HCT116 cells by qRT-PCR, Western and ELISA. The expression of SEMA3F was normalized to the expression in Lv-shSEMA3F cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

leads to reduced apoptosis percentage in SW480 cells (Figure 3A,B). Expression of apoptosis related proteins was tested by Western blot. The results showed that the anti-apoptotic BCL-2 was up-regulated and the pro-apoptotic BAD and BAX were down-regulated in Lv-shSEMA3F cells compared with Lv-control cells (Figure 3C). However, there was no difference in the expression of tumor suppressor P53. Accordingly, knock-down of SEMA3F leads to reduced apoptosis percentage in HCT116 cells (Figure 3D,E), accompanied with up-regulation of BCL-2 and down-regulation of BAD and BAX (Figure 3F). These results demonstrate that knockdown of SEMA3F reduces apoptosis of CRC cells.

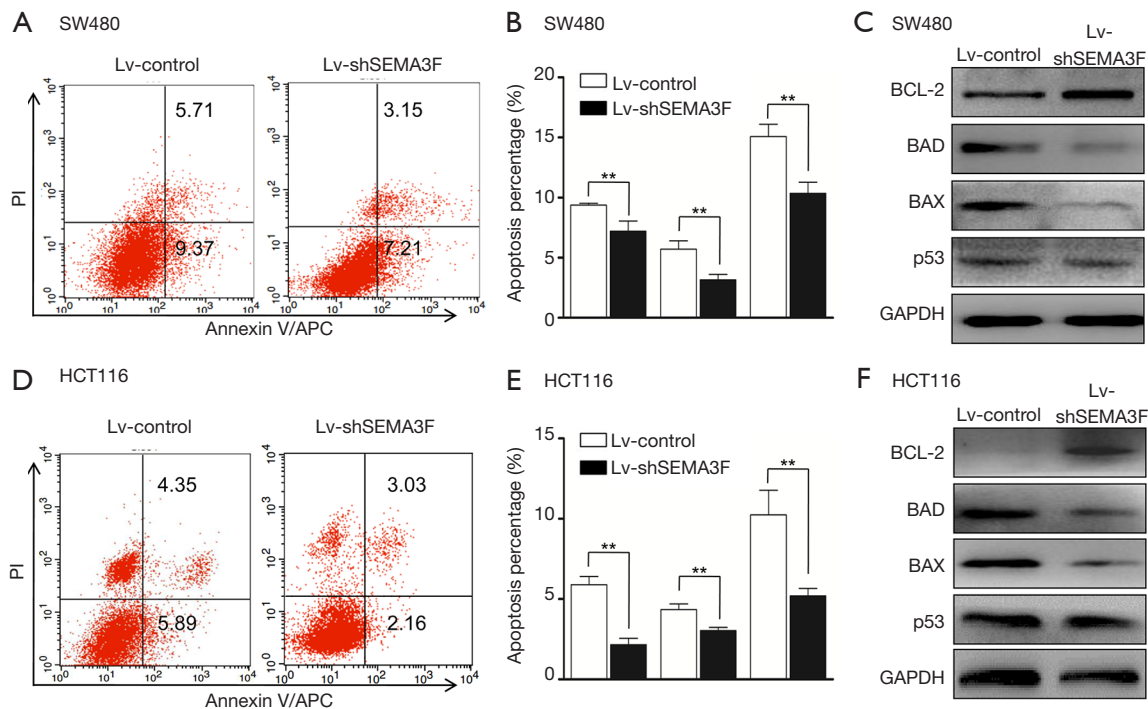
#### EMT features of SEMA3F knock-down cells

Cell invasion assay showed that knock-down of SEMA3F

significantly enhanced invasion capacity of HCT116 and SW480 cells (Figure 4A). As EMT has been recognized as an important process in distant metastasis and therapeutic resistance, the expression of EMT markers was tested by Western blot (Figure 4B,C) and Immunofluorescence (Figure 4D). The results showed that the epithelial marker E-cadherin was down-regulated, while the mesenchymal markers Slug, Snail and Vimentin were up-regulated in Lv-shSEMA3F cells in comparison with Lv-control cells. These results reveal that knock-down of SEMA3F promotes EMT of CRC cells.

#### Attenuated chemosensitivity to oxaliplatin of SEMA3F knock-down cells

Oxaliplatin was the first platinum drug with proven anti-CRC activity and has become a standard in the management



**Figure 3** Reduced apoptosis percentage of SEMA3F knock-down cells. (A) The apoptosis assay showed that the apoptosis percentage was reduced in SW480 cells following knock-down of SEMA3F; (B) the apoptosis percentage was significantly reduced in Lv-shSEMA3F SW480 cells; (C) the expression of apoptosis related proteins was detected using Western blot; (D) the apoptosis percentage in HCT116 cells following knock-down of SEMA3F; (E) the apoptosis percentage was significantly reduced in Lv-shSEMA3F HCT116 cells; (F) the expression of apoptosis related proteins was detected using Western blot. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

of CRC. The existence of intrinsic or acquired drug resistance is a major reason for treatment failure (15). The role of SEMA3F in chemosensitivity to oxaliplatin of CRC cells was evaluated by cell viability assay. The result showed that the  $IC_{50}$  to oxaliplatin of Lv-shSEMA3F cells was dramatically higher than control cells. The  $IC_{50}$  to oxaliplatin of SW480 was increased from 0.54 to 1.31  $\mu M$  following knock-down of SEMA3F (Figure 5A,B;  $P < 0.01$ ). Knock-down of SEMA3F significantly reduced oxaliplatin-induced apoptosis (Figure 5C). In HCT116 cells, the  $IC_{50}$  to oxaliplatin was in 2.54 to 7.43  $\mu M$  following knock-down of SEMA3F (Figure 5D,E;  $P < 0.01$ ). In addition, knock-down of SEMA3F significantly reduced oxaliplatin-induced apoptosis in HCT116 cells (Figure 5F). In summary, these results demonstrate that knock-down of SEMA3F decreases chemosensitivity to oxaliplatin of CRC cells.

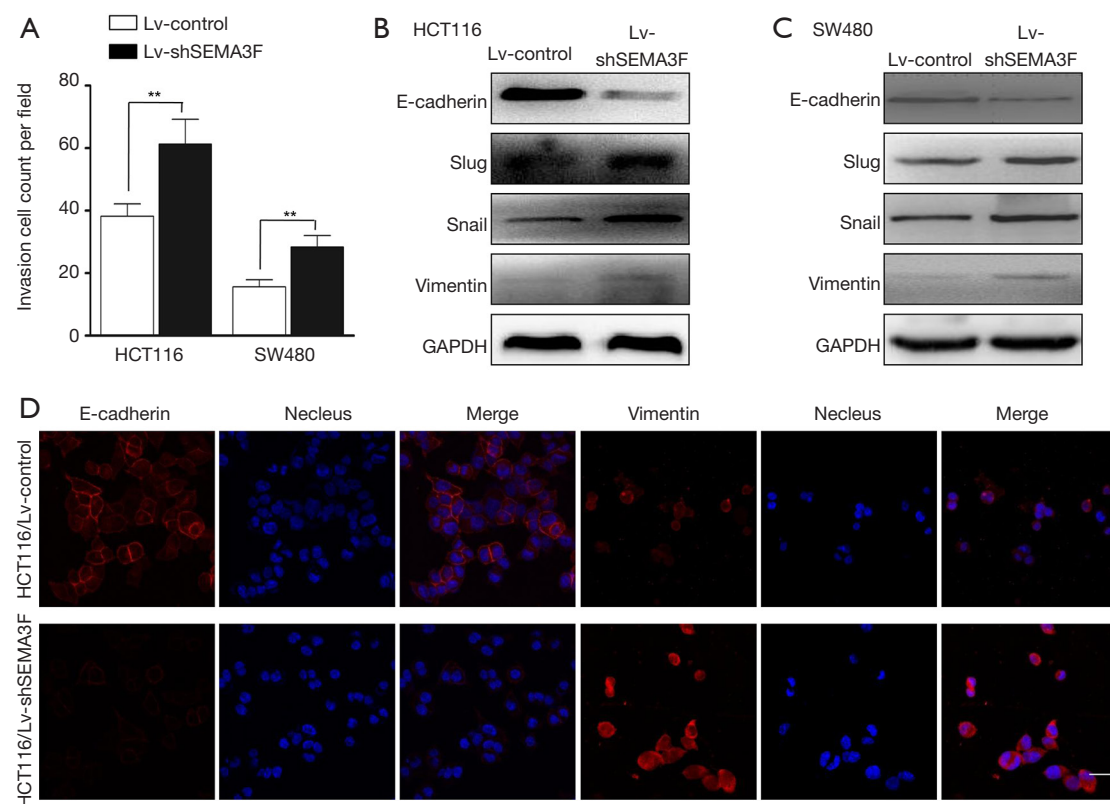
#### Decreased therapeutic effect of oxaliplatin on SEMA3F knockdown cells *in vivo*

We finally test the role of SEMA3F in the management of

CRC with oxaliplatin *in vivo* using orthotopic xenograft model. Three weeks after transplantation, mice were treated with oxaliplatin 6 mg/kg/week i.p. or PBS. The volume of tumors originated from Lv-shSEMA3F cells was larger than that from Lv-control cells (Figure 6A,B). Although treatment with oxaliplatin reduced the tumor volume significantly, the tumor volume of Lv-shSEMA3F cells was still larger than that of Lv-control cells. Moreover, the overall survival time of mice transplanted with Lv-shSEMA3F cells was shorter than that of those with Lv-control cells. Under treatment with oxaliplatin, the overall survival time of mice transplanted with Lv-shSEMA3F cells was still shorter (Figure 6C). Taken together, these results revealed that knock-down of SEMA3F attenuates therapeutic effect of oxaliplatin *in vivo*.

#### Discussion

Although originally recognized as axon guidance repulsive, SEMA3F has been shown to inhibit the progression of tumor. Bielenberg reported that SEMA3F blocked metastases



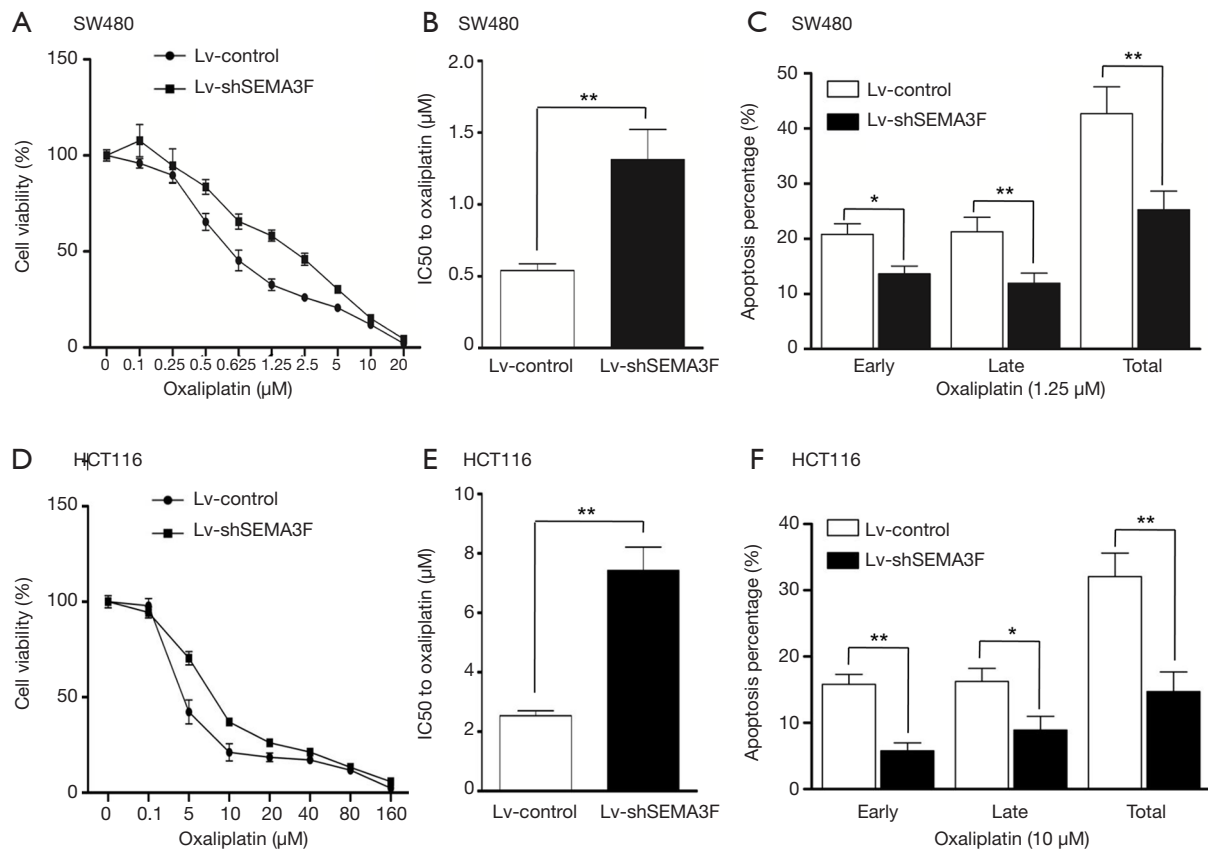
**Figure 4** EMT features of SEMA3F knock-down cells. (A) The invasion assay showed that the invasion and migration capacity of Lv-shSEMA3F cells was enhanced; (B) the expression of EMT markers in HCT116 cells was detected using Western blot; (C) the protein abundance of EMT markers in SW480 cells; (D) immunofluorescence assay showing decreased expression of E-cadherin and increased expression of Vimentin in Lv-shSEMA3F HCT116 cells. Scale bar, 50  $\mu$ m. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

of melanoma cells transplanted subcutaneously into nude mice and those tumors were poorly vascularized (10). Over-expression of SEMA3F significantly prolonged survival of nude rats orthotopically transplanted with lung cancer cells, accompanied with loss of activated  $\alpha\beta 3$  integrins (23). Further study showed that SEMA3F affects multiple signaling pathways in lung cancer cells, including reducing AKT and STAT phosphorylation and integrin-linked kinase activity (24). SEMA3F also had a repulsive effect on motile breast cancer cell line C100 (25). In addition, SEMA3F has been reported to reduce colony formation, adhesion and invasion of ovarian cancer cells, which was associated with reduced phosphorylation of focal adhesion kinase and down-regulation of matrix metalloproteinase-2/9 (26). Our previous work found that down-regulation of SEMA3F was related to short overall survival time and increased distant metastasis (9). More recently, SEMA3F has been reported to inhibit proliferation and metastasis of ileal neuroendocrine

tumor cells at least partially through inactivating mTOR and MAPK pathways (27).

The present work found that the expression of SEMA3F is negatively correlated with the expression of P-gp and GST- $\pi$  in CRC tissues from 94 patients. Both P-gp and GST- $\pi$  are involved in the chemoresistance of cancer cells. P-gp, also called ABCB1 or MDR1, is a member of the ABC transporter family, which pumps chemotherapeutic agents out of cancer cells (28). GST- $\pi$  belongs to the Glutathione S-transferases family, which catalyze the conjugation of toxic and carcinogenic electrophilic molecules with GSH to protect macromolecules from damage (29). High expression of GST- $\pi$  has been reported in colon cancer and in drug-resistant tumors (15). We for the first time report that knock-down of SEMA3F reduces apoptosis and chemosensitivity to oxaliplatin of CRC cells *in vitro*. Using orthotopic xenograft model, we found that knock-down of SEMA3F attenuated therapeutic effect of oxaliplatin *in*



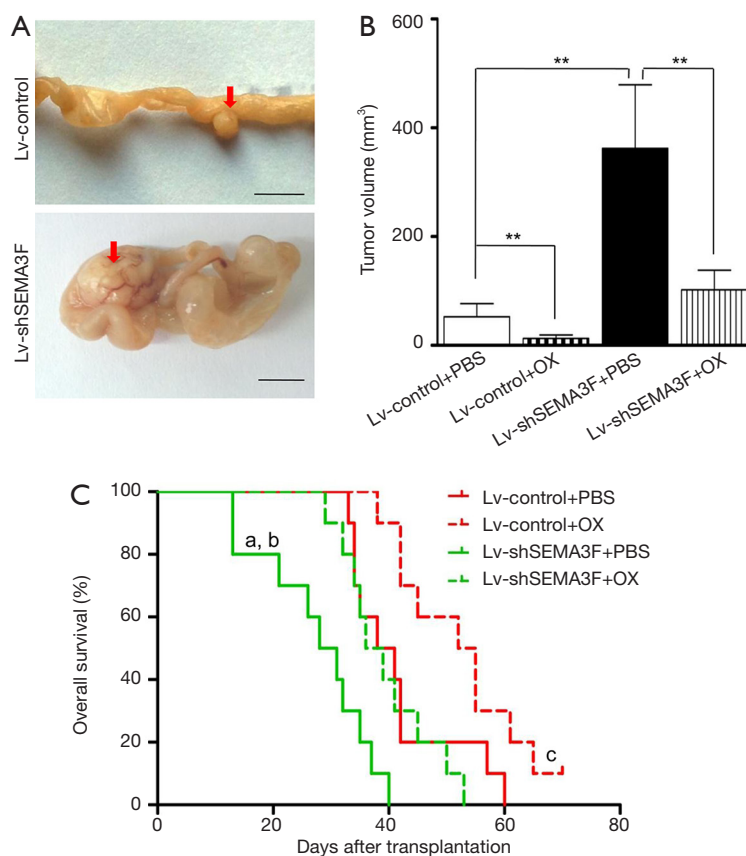


**Figure 5** Attenuated chemosensitivity to oxaliplatin of SEMA3F knock-down cells. (A) The cell viability of SW480 cells detected by CCK-8 after the treatment with oxaliplatin at the indicated concentrations for 72 hours; (B) the IC<sub>50</sub> to oxaliplatin of Lv-shSEMA3F SW480 cells was significantly larger than that of Lv-control cells; (C) the apoptosis percentage following the treatment with oxaliplatin was determined by apoptosis assay; (D) the cell viability of HCT116 cells detected by CCK-8 after the treatment with oxaliplatin at the indicated concentrations for 72 hours; (E) the IC<sub>50</sub> to oxaliplatin of Lv-shSEMA3F HCT116 cells was significantly larger than that of Lv-control cells; (F) the apoptosis percentage following the treatment with oxaliplatin was determined by apoptosis assay. \*, P<0.05; \*\*, P<0.01.

*in vivo*. In consistence with previous studies, we found that knock-down of SEMA3F increased the vascular density in orthotopic xenografts (data not shown), which may decrease the delivery of oxaliplatin and thus influence the efficiency of oxaliplatin. Moreover, it has been reported that SEMA3F can bind the Neuropilin-2 receptor (30). The direct effect of VEGF on tumor cells remains poorly known until Bhattacharya *et al.* reported that the intracellular but not the extracellular VEGF elevated cell viability of CRC cells by activating PI3K/mTOR-AKT signaling (31). As a secreted molecule, SEMA3F activates down-stream signaling through binding to the cell membrane receptors, including Neuropilin-2 and PlexinA1 (1). Therefore, competing with VEGF for binding Neuropilin-2 could not contribute to the pro-apoptosis effect of SEMA3F. On the other hand, the

PI3K-AKT signaling, which can be inhibited by SEMA3F, plays fundamental role in survival, growth, invasion and metastasis of cancer cells (32). We therefore propose that SEMA3F exerts its pro-apoptosis effect through inhibiting PI3K-AKT signaling.

Oxaliplatin is the third-generation platinum drug that is used for the treatment of CRC, gastric and pancreatic cancers. Since its introduction in the treatment of metastatic CRC in 2000, oxaliplatin has largely increased objective response rates, percentage of metastasis resection and overall survival of patients (15). Unfortunately, treatment failure occurs mainly due to intrinsic or acquired resistance. The molecular mechanisms associated with oxaliplatin resistance include intracellular transport and detoxification, alterations in DNA repair mechanisms and cell death mechanisms.



**Figure 6** Decreased therapeutic effect of oxaliplatin on SEMA3F knockdown cells *in vivo*. (A) Representing images of tumors originated from Lv-control cells or Lv-shSEMA3F HCT116 cells; (B) the volume of tumors originated from Lv-control cells or Lv-shSEMA3F cells with or without the treatment with oxaliplatin (OX); (C) the overall survival of mice injected with Lv-control cells or Lv-shSEMA3F cells with or without the treatment with oxaliplatin (OX). a, versus 'Lv-control+PBS',  $P < 0.01$ ; b, versus 'Lv-shSEMA3F+OX',  $P < 0.01$ ; c, versus 'Lv-control+PBS'. Scale bar: 0.5 cm.  $P < 0.05$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.01$

Failure in the oxaliplatin induced DNA damage repair usually caused cell death activation. Therefore, the activation of cell death pathways influences the efficacy of oxaliplatin. Oxaliplatin exert its cytotoxic effect mainly by activating the intrinsic apoptosis pathway (33). The intrinsic apoptosis pathway is regulated by the BCL-2 family, which contains pro-apoptotic (BAD, BAX, BAK) and anti-apoptotic members (BCL-2, BCL-xl, MCL-1). It has been reported that loss of pro-apoptotic BAX decreases while down-regulation of the anti-apoptotic BCL-2 and BCL-xl increases sensitivity to oxaliplatin (34,35). Consistently, we found that the anti-apoptotic BCL-2 was up-regulated and the pro-apoptotic BAD and BAX was down-regulated in Lv-shSEMA3F cells compared with Lv-control cells. However, there was no difference in the expression of P53. Moreover, we found that knock-down

of SEMA3F increased promotes invasion and EMT of CRC cells. It should be noticed that the increased invasion of CRC cells might be a combined effect of the elevated invasion capacity and cell viability. During the process of EMT, cells undergo loss of epithelial cell-cell junctions, re-organization of cytoskeleton and molecular changes such as decrease the expression of epithelial markers and increase the expression of mesenchymal markers. Besides invasion, metastasis and stemness, EMT also plays important role in drug resistance in many cancers, such as breast cancer, lung cancer, pancreatic cancer and CRC (18,36). Many signals in the tumor microenvironment, such as oxygen tension, cytokines and growth factors can induce EMT in cancer (37). We propose that SEMA3F increases chemosensitivity to oxaliplatin of CRC cells at least partially through activating apoptosis pathways and inhibiting the EMT process.

In conclusion, the present study demonstrated that SEMA3F induces apoptosis, inhibits EMT and increases chemosensitivity to oxaliplatin of CRC cells. These results provides evidence for the development of novel therapeutic intervention of CRC based on the axon guidance repulsive SEMA3F.

### Acknowledgments

**Funding:** This work was supported by the National Natural Science Foundation for Young Scientist of China (No. 81602097, 81402460) and Key research of the 309th hospital (No.2016ZD-009). We thank for the unselfish help from Pro. Xiu-Wu Bian (Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, China).

### Footnote

**Conflicts of Interest:** All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2017.01.15>). The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was carried out in according to the principles of the Helsinki Declaration and approved by the Ethical Committee of the Third Military Medical University (No. 2013062). Written informed consent was obtained from all patients.

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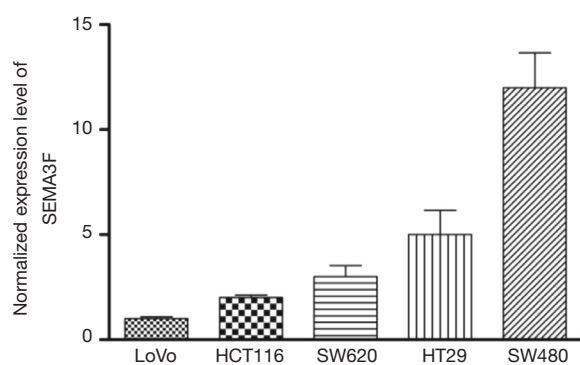
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**Cite this article as:** Fan M, Yang L, Li F, Sun YM, Zhou ZH, Guan JZ. Axon guidance repulsant SEMA3F increases chemosensitivity to oxaliplatin and inhibits epithelial-mesenchymal transition of colorectal cancer cells. *Transl Cancer Res* 2017;6(1):206-217. doi: 10.21037/tcr.2017.01.15



## Supplementary



**Figure S1** The expression of Semaphorin-3F (SEMA3F) in colorectal cancer (CRC) cell lines. The expression of SEMA3F in CRC cells was determined by RT-PCR, which showed highest expression of SEMA3F in SW480 cells and lowest in LoVo cells.