SEMA3B improves the survival of patients with esophageal squamous cell carcinoma by upregulating p53 and p21

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Abstract. As one of the most common malignancies, esophageal squamous cell carcinoma (ESCC) is ranked as the sixth leading cause of cancer-related death worldwide. In our previous study, by employing cDNA microarray analysis, semaphorin 3B (SEMA3B) was found to be significantly downregulated in ESCC. In the present study, SEMA3B downregulation at the mRNA level was found in 34 of 60 primary ESCCs (56.7%) and in 6 of 9 ESCC cell lines (66.7%) by transcription-polymerase chain reaction (RT-PCR). Moreover, immunohistochemical (IHC) staining of SEMA3B in a tissue microarray further indicated that downregulated expression of SEMA3B protein was found in 125 of 222 (56.3%) ESCC cases and downregulation of SEMA3B protein was significantly correlated with lymph node metastasis (P=0.000), advanced clinicopathological stage (P=0.001) and poor disease-specific survival (P=0.017) of ESCC patients. In addition, functional studies demonstrated that the SEMA3B gene could suppress the tumorigenic ability of ESCC cells and cell motility. Furthermore, it was found that by upregulating p53 and p21 expression and inhibiting Akt (Ser473) phosphorylation, SEMA3B could induce cell cycle arrest at G1/S phase. Taken together, our results suggest that SEMA3B may be an important tumor-suppressor gene in the malignant progression of ESCC, as well as a valuable prognostic marker for ESCC patients.

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Introduction

Characterized by high mortality and poor prognosis, esophageal cancer is one of the most common malignant tumors worldwide, and the 5-year survival rate of advanced stage esophageal squamous cell carcinoma (ESCC) patients is only 10%. In China, ESCC is the main histologic subtype, and the Henan Province of Northern China, Linzhou and its adjacent counties have the highest incidence of ESCC worldwide (1,2). Furthermore, previous studies have revealed that ESCC development is closely associated with various risk factors, such as heredity, environment and diet (3). Such as other solid tumors, the occurrence of ESCC is a long process with numerous genetic alterations, including oncogene activation and tumor-suppressor gene inactivation. As detected by comparative genome hybridization and loss of heterozygosity, 3p deletion is one of the most common genetic changes in ESCC, therefore there may be one or more tumor-suppressor genes in 3p (4-7). A number of genes located in 3p have already been considered as candidate tumor-suppressor genes in ESCC, such as FHIT, RASSF1A, CACNA2D2, DLEC1 and PLCδ1 (8-11).

As a member of the semaphorin family, semaphorin 3B (SEMA3B) is located in the 3p21.3 region. SEMA3B encodes a secreted protein consisting of 749 amino acids, including a signal peptide, a highly conservative Sema structure which has an immune globulin structure domain. Identified as a candidate tumor-suppressor gene, SEMA3B exhibits frequent allelic loss and downregulation in lung and breast cancer, ovarian and hepatocellular carcinoma and cholangiocarcinomas (12-15). However, the relationship between SEMA3B and ESCC occurrence and development has not been explored. In our previous study, SEMA3B was found to be frequently downregulated in ESCC specimens, which was detected by cDNA microarray analysis (16). In the present study, reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical (IHC) staining were employed to determine SEMA3B expression at the mRNA and protein levels in clinical ESCC samples. Moreover, *in vitro* assays were adopted to study the tumor-suppressive function of SEMA3B. In addition, the tumor-suppressive mechanism of SEMA3B and its clinical significance in ESCC were investigated as well.

Materials and methods

Cell lines and primary tumor specimens. Three Chinese ESCC cell lines (HKESC1, EC18 and EC109), six Japanese ESCC cell lines (KYSE30, KYSE140, KYSE180, KYSE410, KYSE510 and KYSE520; reviewed in ref. 17) and two immortalized esophageal epithelial cell lines (NE1 and NE3) were kindly provided by Professor Srivastava (Department of Pathology, University of Hong Kong). After surgical resection at Linzhou Cancer Hospital, a total of 300 primary ESCC tumor tissue samples and their paired non-tumorous tissue samples were immediately collected from the proximal resection margins. Some were quickly put into vials and stored in liquid nitrogen and other tumor tissues were routinely formalin-fixed and paraffin-embedded. Before operative treatment, no patients recruited in the study received chemotherapy or radiotherapy and the follow-up period was 60 months. Samples used in the present study were approved by the Individual Institutional Committees for Ethical Review of Research Involving Human Subjects.

Semi-quantitative reverse transcription-PCR. Through the application of TRIzol (Invitrogen, Carlsbad, CA, USA), total RNA was extracted from the cell lines and frozen ESCC tissues. Advantage RT for PCR kit (Clontech, Mountain View, CA, USA) was employed to conduct reverse transcription of total RNA (2 μg) and cDNA was subjected to PCR for 30 cycles of amplification with the following pairs of primers: SEMA3B Fw, 5'-CCAGTGCCAAGAGGCGGTTC and SEMA3B Rv, 5'-AGCACCTGGGTGTGGGCTGT. The GAPDH gene served as a control with the pair of primers: GAPDH Fw, 5'-CGGGAAGCTTGTCATCAATGG and GAPDH Rv, 5'-GGCAGTGATGGCATGGACTG. In order to analyze the relative grey value, the mRNA expression was quantified by Quantity One software, during which GAPDH was regarded as an internal control.

Tissue microarrays and immunohistochemistry. As previously described, tissue microarrays (TMA) containing 300 pairs of primary ESCC tissue samples and their corresponding non-tumorous tissues were constructed (19). In short, tissue sections with a thickness of 5 μm were cut from tissue micro-array blocks and mounted on microscope slides. By adopting the standard streptavidin-biotin-peroxidase complex method, immunohistochemistry (IHC) was performed (19). TMA slides were deparaffinized in xylene, rehydrated through a graded alcohol series and incubated with 3% hydrogen peroxide. For antigen retrieval, TMA slides were boiled in 10 mM sodium citrate buffer (pH 6.0) for 15 min with the help of a pressure cooker. Blocked by 10% normal rabbit serum at room temperature for 30 min, the slides were then incubated with primary anti-SEMA3B monoclonal antibody (1:50 dilution; Lifespan) at 4°C overnight. At a concentration of 1:75, the TMA sections were incubated with biotinylated goat anti-rabbit immunoglobulin for 30 min at 37°C. Through the application of ImmPRESS™ Universal Antibody kit, primary antibody staining was visualized with NovaRed (both from Vector Laboratories, Burlingame, CA, USA) as a substrate. In the next step, the sections were counterstained with hematoxylin, dehydrated and mounted. As previously

described, an immunoreactivity scoring (IRS) system was applied (19). The percentage of SEMA3B-positive cells was scored as 0, <5%, negative; 1, 5-25%, sporadic; 2, >25-50%, focal; 3, >50%, diffuse; and the intensity of SEMA3B-positive staining was scored as 0, negative; 1, weak; 2, moderate; and 3, strong. Both the percentage of positive cells and cell staining intensity were determined in a double-blinded manner. According to the following formula, the total score was determined: Staining index = intensity x positive rate. In the present study, a staining index of ≤4 was considered to be downregulated expression and a staining index of >4 was considered as normal expression.

SEMA3B-30-KYSE30 (SEMA3B-30) esophageal cancer cell line construction with SEMA3B overexpression. In order to test the function and mechanism underlying growth inhibition by SEMA3B, SEMA3B was PCR amplified, sequence-verified, cloned into pcDNA3.1(+) vector (Invitrogen) and transfected into ESCC, KYSE30 cells which lacked SEMA3B expression. A stable SEMA3B-expressing clone was selected and SEMA3B cDNA was re-sequenced. Blank vector-transfected KYSE30 cells (Vec-30) were used as control. RT-PCR and western blot analysis were employed to detect SEMA3B expression. Taking GAPDH as an internal control, mRNA and protein expression was quantified by Quantity One software, to analyze the relative grey value.

Tumor-suppressive function of SEMA3B. In order to test the tumor-suppressive function of the SEMA3B gene, foci formation assay was performed. Into a 6-well plate, 1×10^3 SEMA3B-30 and Vec-30 cells were planted. After being cultured for 10 days, surviving colonies (>50 cells/colony) were counted with Giemsa staining and triplicate independent experiments were carried out. Moreover, cell growth rates of the SEMA3B-30 and Vec-30 cells were detected by MTT assay. At a density of 1×10^3/well, the cells were seeded in a 96-well plate. According to the manufacturer's instructions, the cell growth rate was aassessed using cell proliferation MTT kit (Sigma). The experiment was conducted in triplicate.

Migration and invasion assays. For the cell migration assays, SEMA3B-30 or Vec-30 cells were cultured in a 6-well plate until confluent and the cell layer was wound using a sterile tip. After being incubated for 36 h, the cells were photographed under a phase-contrast microscope. The experiment was conducted in triplicate. For the invasion assay, SEMA3B-30 or Vec-30 cells were starved of serum-free medium for 24 h before the assay. After suspension in 500-μl serum-free medium, the cells (5×10^4) were loaded onto the upper compartment of an invasion chamber coated with Matrigel (BD Biosciences). However, the lower compartment was filled with 500-μl complete medium as chemoattractant. After 48 h, the invasive cells were fixed, stained and counted, and independent experiments were carried out in triplicate.

Cell cycle analysis. SEMA3B-30 or Vec-30 cells (1×10^6) were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). When the cells reached 70% confluency, the serum was withdrawn from the culture medium. After 72 h, 10% FBS was added to the medium for an additional 12 h. The
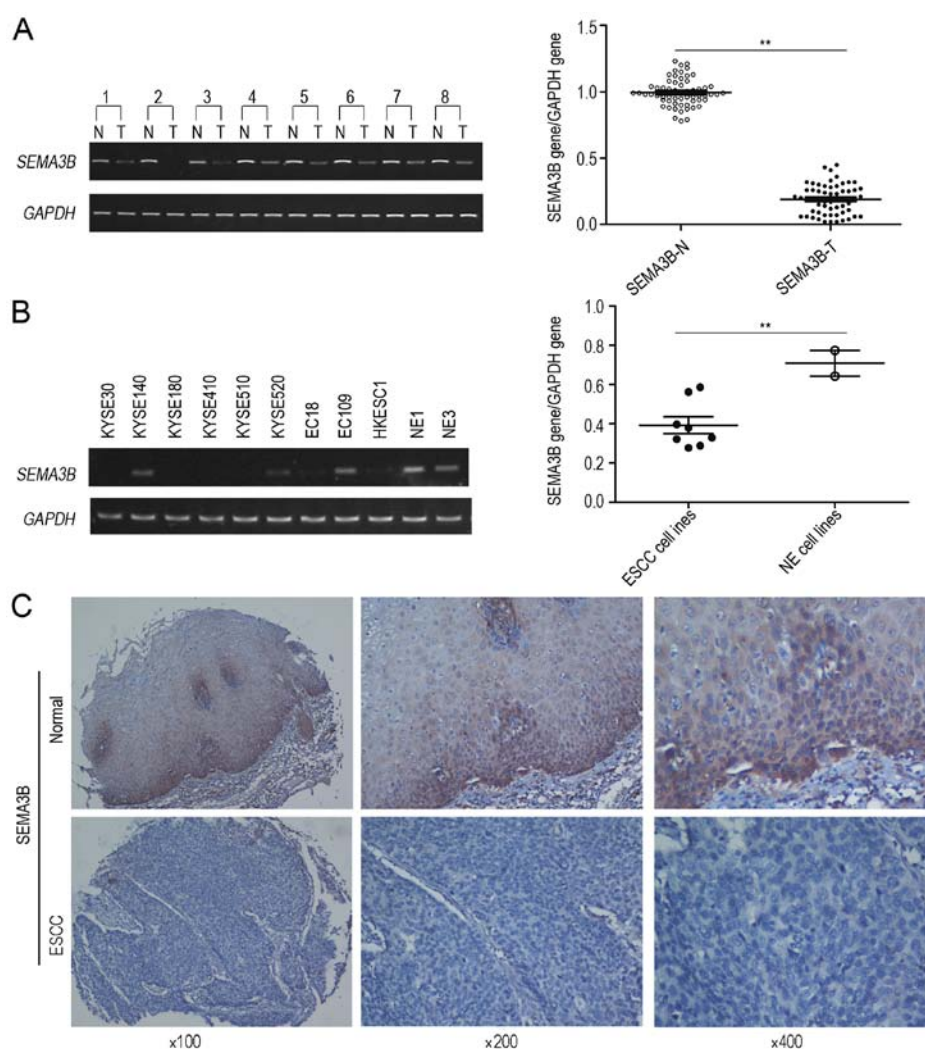
Figure 1. Downregulation of SEMA3B in ESCC. (A) The relative SEMA3B mRNA expression in tumor tissues (T) was frequently downregulated in 60 ESCCs in comparison with their paired non-tumor (N) tissues by RT-PCR. *GAPDH* was used as an internal control. Values are the mean ± SD of 3 independent experiments; [**]P<0.05. (B) The relative SEMA3B mRNA expression in ESCC cell lines was frequently downregulated in comparison with two immortalized esophageal epithelial cell lines including NE1 and NE3 by RT-PCR. *GAPDH* was used as an internal control. Values are the mean ± SD of 3 independent experiments; [**]P<0.05. (C) Representative images of SEMA3B expression in a pair of ESCC (lower panels) and adjacent normal tissues (upper panels) as detected by immunostaining with the anti-SEMA3B antibody (brown). The slides were counterstained with hematoxylin. Original magnification, x100 (left), x200 (middle) and x400 (right).

cells were fixed in 70% ethanol and stained with propidium iodide. In addition, DNA content was analyzed by applying Cytomics FC (Beckman Coulter).

Western blot analysis. SEMA3B-30 and Vec-30 cell lysates were obtained. Followed by protein transfer to polyvinylidene fluoride membranes (PVDF), equal amounts of protein from each sample were diluted with loading buffer, denatured and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being incubated in a blocking solution containing 5% non-fat milk powder in Tris-buffered saline and Tween-20 (TBST) buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature, the membranes were immunoblotted overnight with primary monoclonal antibodies for GAPDH, p53, p21, cyclin D1, total Akt and Akt (Ser473; Cell Signaling Technology) at a dilution of 1:1,000 at 4˚C and then incubated with the secondary antibody (1:1,000 dilution) for 2 h at room

temperature. Enhanced chemiluminescence detection system was adopted to detect the protein antibody complex. The protein expression was quantified by Quantity One software, so as to analyze relative grey value, during which GAPDH served as an internal control.

Statistical analysis. SPSS standard version 16.0 was used to carry out the statistical analysis. Correlations between SEMA3B expressions and clinicopathological characteristics were assessed by χ^2 or Fisher's exact tests. In addition, disease-specific survival (DSS) was calculated from the date of diagnosis to the date of cancer-related death or last follow-up. According to the Kaplan-Meier method, survival curves were generated and statistical analysis was performed by making use of the log-rank test. However, the Cox proportional hazards regression model was employed to identify independent prognostic factors. A P-value of <0.05 was considered to indicate a statistically significant result.

Table I. Association of the downregulation of SEMA3B expression with clinicopathological characteristics of the patients with ESCC (n=222).

Clinicopathological characteristics	n	SEMA3B expression, n (%)		χ^2	P-value
		Downregulated expression	Normal expression		
Age (years)					
≤60	102	58 (56.9)	44 (4.1)	0.024	0.893
>60	120	67 (55.8)	53 (44.2)		
Gender					
Male	126	70 (55.6)	56 (44.4)	0.067	0.891
Female	96	55 (57.3)	41 (42.7)		
Tumor cell differentiation					
Well	29	15 (51.7)	14 (48.3)	0.290	0.865
Moderate	146	83 (56.8)	63 (43.2)		
Poor	47	27 (57.4)	20 (42.6)		
Lymph node metastasis (N)					
N0	125	55 (44.0)	70 (56.0)	1.76E1	0.000[a]
N1	97	70 (72.2)	27 (27.8)		
TNM stage					
I	12	3 (25.0)	9 (75.0)	1.49E1	0.001[a]
II	134	67 (50.0)	67 (50.0)		
III	76	55 (72.4)	21 (27.6)		
General classification					
Medullar type	120	64 (53.3)	56 (46.7)	1.316	0.725
Ulcerative type	65	39 (60.0)	26 (40.0)		
Sclerotic type	12	8 (66.7)	4 (33.3)		
Mushroom type	25	14 (56.0)	11 (44.0)		

[a]$P<0.05$. SEMA3B, semaphorin 3B; ESCC, esophageal squamous cell carcinoma; TNM, tumor-node-metastasis.

Results

Frequent downregulation of SEMA3B in ESCCs. Our previous study showed that SEMA3B was significantly downregulated in ESCC by cDNA microarray (16), thus the expression of SEMA3B at the mRNA and protein levels was explored in the present study. The expression of SEMA3B at the mRNA level was assessed by semi-quantitative RT-PCR in 60 primary ESCC tumors as well as their paired non-tumorous tissues and 9 ESCC cell lines. The results showed that the downregulation of SEMA3B was detected in 34/60 (56.7%) ESCC tumor tissues in comparison with their paired non-tumorous tissues. Ratios of SEMA3B gene/GAPDH expression for ESCC tissues and their paired non-tumor tissues were 0.19±0.11 and 0.99±0.10, respectively (Fig. 1A). In comparison with the non-tumor tissues, lower expression of SEMA3B (P<0.05) was found in the ESCC tissues. Meanwhile, the downregulation of the expression of SEMA3B was detected in 6/9 (66.7%) (EC18, HYESC1, KYSE30, KYS180, KYS410 and KYS510) ESCC cell lines. Ratios of SEMA3B gene/GAPDH expression for ESCC cell lines and immortalized esophageal epithelial cell lines were 0.39±0.12 and 0.71±0.09, respectively (P<0.05; Fig. 1B). Furthermore, the quantity of samples was expanded to verify its expression at the protein level. A consistent result

was detected in the expression of SEMA3B at the protein level by IHC through application of TMA. Informative results were obtained from 222 pairs of ESCCs. Non-informative samples including lost samples, unrepresentative samples, samples with too few tumor cells, and samples with inappropriate staining were not involved in data analysis. Downregulation of SEMA3B at the protein level was found in 125/222 (56.3%) informative ESCC cases (Fig. 1C).

Clinical significance of SEMA3B downregulation in ESCC. Afterwards, we examined the clinical association between the expression of SEMA3B protein and the clinicopathological characteristics of the ESCC cases. The analysis demonstrated that downregulation of SEMA3B protein was significantly correlated with lymph node metastasis (P=0.000) and advanced clinical stage (P=0.001; Table I), but was not correlated with gender, age, cell differentiation and general classification. The univariate survival analysis revealed that downregulation of SEMA3B, poor differentiation, status quo of lymph node metastasis and advanced tumor-node-metastasis (TNM) stage were significantly correlated with poor overall survival (P<0.05; Table II). All these variables which showed statistical significance in the univariate analysis were further examined by multivariate analysis (Table III). The results revealed that
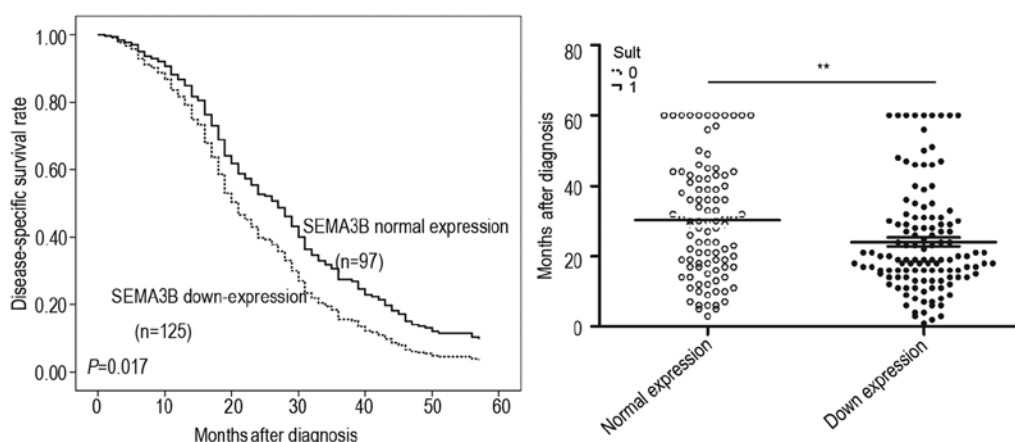
Figure 2. Kaplan-Meier analysis of survival in patients with ESCC according to SEMA3B expression. Solid line indicates patients with normal SEMA3B expression (n=97, median survival of 19 months); dotted line indicates patients with downregulation of SEMA3B (n=125, median survival of 37 months; P=0.017, log-rank test).

Table II. Univariate Cox regression analysis of factors possibly influencing disease-specific survival in patients with ESCC.

Variables	Hazard ratio	95% CI	P-value
SEMA3B expression	0.697	0.527-0.924	0.012[a]
Age (years)	1.263	0.958-1.667	0.098
Gender	1.052	0.794-1.392	0.725
Tumor cell differentiation	1.826	1.159-2.877	0.009[a]
pN factor	1.697	1.284-2.243	0.000[a]
TNM stage	2.093	1.563-2.802	0.000[a]

[a]P<0.05. ESCC, esophageal squamous cell carcinoma; CI, confidence interval; SEMA3B, semaphorin 3B; TNM, tumor-node-metastasis.

Table III. Multivariate Cox regression analysis of factors possibly influencing disease-specific survival in patients with ESCC.

Variables	Hazard ratio	95% CI	P-value
SEMA3B expression	0.698	0.520-0.938	0.017[a]
Tumor cell differentiation	1.944	1.227-3.082	0.005[a]
pN factor	0.897	0.536-1.500	0.679
TNM stage	2.143	1.257-3.653	0.005[a]

[a]P<0.05. ESCC, esophageal squamous cell carcinoma; CI, confidence interval; SEMA3B, semaphorin 3B; TNM, tumor-node-metastasis.

SEMA3B downregulation was an independent risk factor for overall patient survival (P=0.017), tumor cell differentiation (P=0.005) and TNM stage (P=0.005). The results of Kaplan-Meier analysis demonstrated that ESCC patients with

SEMA3B downregulation (median survival time, 19 months) had a shorter disease-specific survival (DSS) than patients with normal SEMA3B expression (median survival time, 30 months; P=0.017; Fig. 2).
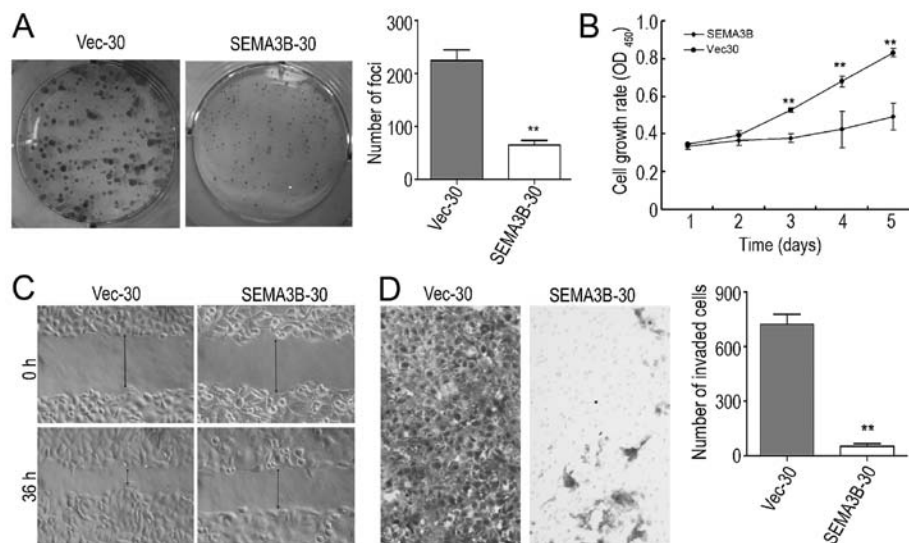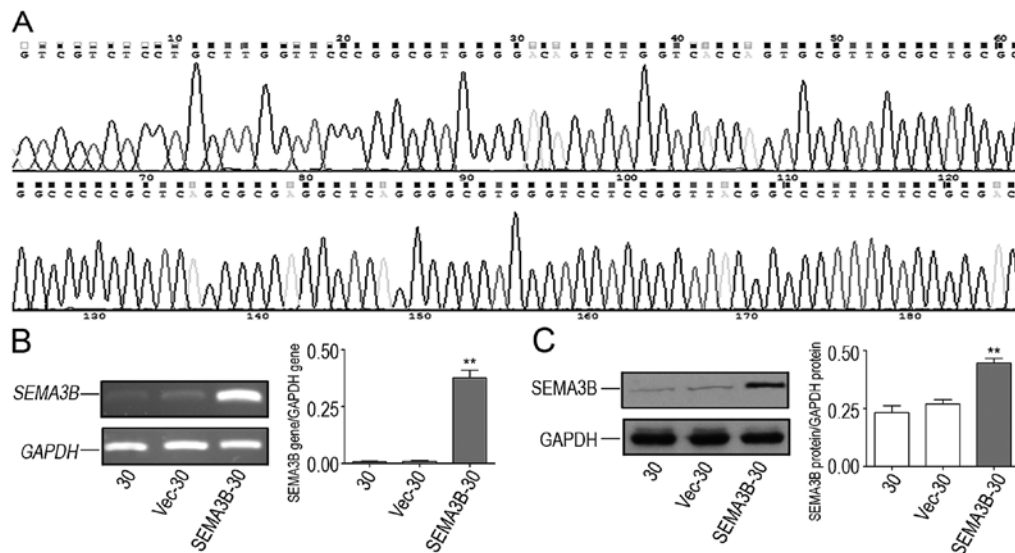
Establishment of a stable SEMA3B-expressing cell line. The pcDNA3.1(+)-SEMA3B plasmid was constructed and its sequence was subsequently confirmed (Fig. 3A). Both the two plasmids, pcDNA3.1(+)-SEMA3B and blank vector were transfected into KYSE30 cells separately. In order to detect the transfection efficiency of the plasmids in the SEMA3B-30 cells, the SEMA3B gene and protein expression in the SEMA3B-30 cells were confirmed by RT-PCR and western blot analysis. Ratios for SEMA3B gene/GAPDH gene expression for the KYSE30, Vec-30 and SEMA3B-30 cells were 0.0068±0.0047, 0.0084±0.0069 and 0.3766±0.0574, respectively (Fig. 3B; P<0.05). Rates for SEMA3B protein/GAPDH protein expression were 0.2321±0.0510, 0.2691±0.0333 and 0.4460±0.0343, respectively (Fig. 3C; P<0.05). In comparison with the KYSE30 and blank vector-transfected KYSE30 (Vec-30) cells, the expression of SEMA3B was higher in the SEMA3B-30 cells.

SEMA3B blocks proliferation and migration of ESCC cells. The tumor-suppressive function of SEMA3B was assessed by foci formation and cell growth assays. The mean value of the colony formation quantity of the SEMA3B-30 cells was 55.5±10.60, which was lower than that of the Vec-30 cells (220±46.32). The foci formation assay showed that the efficiency of foci formation was highly inhibited (P<0.05) in the SEMA3B-30 cells in comparison with the Vec-30 cells (Fig. 4A). As confirmed in the cell growth assay, the cell proliferation rate in the SEMA3B-30 cells was significantly inhibited by SEMA3B (P<0.05) in comparison with the Vec-30 cells (Fig. 4B) after five successive days of the measurement of OD values.

The TMA results indicated that the downregulation of the protein expression of SEMA3B was significantly correlated with lymph node metastasis, thus the effects by SEMA3B on cell migration and invasion were studied by wound-healing

Figure 3. Establishment of a stable SEMA3B-expressing cell line. (A) The confirmatory sequence of the SEMA3B gene was re-sequenced. The relative expression of SEMA3B in the SEMA3B-transfected ESCC cells (SEMA3B-30) was confirmed by (B) RT-PCR and (C) western blot analysis. KYSE30 and empty vector-transfected ESCC (Vec-30) cells were used as controls. *GAPDH* was used as an internal control. Values are the mean ± SD of 3 independent experiments; **P<0.05.

Figure 4. Tumor-suppressive function of SEMA3B in ESCC cells. (A) Representative inhibition of foci formation in monolayer culture by SEMA3B and quantitative analyses of foci quantity are shown. Columns indicate means of at least 3 independent experiments; bars, SD. **P<0.05 vs. Vec-30 cells using the Student's t-test. (B) Growth curve of SEMA3B-expressing cells was compared with Vec-30 cells by MTT assay. Data points indicate the mean of at least 3 independent experiments; bars, SD; **P<0.05. (C) Effects of SEMA3B on cell migration were determined by wound-healing assay. Within 36 h, the rate of spreading of SEMA3B-expressing cells along the wound edge was slower than that in the control Vec-30 cells. (D) The SEMA3B-30 and Vec-30 cells that invaded through the Matrigel are shown in representative images. The number of invaded tumor cells was quantified in the right histogram. Columns indicate means of triplicate experiments; **P<0.05.

and cell invasion assays. The wound-healing assay indicated that SEMA3B inhibited cell mobility (Fig. 4C). The Matrigel invasion assay also showed that the number of migrating SEMA3B-30 cells (75±30.61) was lower than that of the Vec-30 cells (768.33±94.73; Fig. 4D). SEMA3B inhibited ESCC cell invasiveness, as the number of invasive SEMA3B-30 cells was significantly decreased in comparison with the Vec-30 cells (P<0.05).

SEMA3B arrests the cell cycle at the G1/S checkpoint by upregulating p21 and p53 and inhibits the phosphorylation of

Akt. To explore the mechanism underlying the growth inhibition of SEMA3B, flow cytometry was used to compare the cell cycle distribution in the SEMA3B-30 cells and the control Vec-30 cells. After 3 days of serum starvation followed by addition of 10% serum for 12 h, significant G1/S phase arrest was found in the SEMA3B-30 cells in comparison with the control Vec-30 cells. The percentage of SEMA3B-30 cells in the G1 phase was 86.35%, while this percentage in the Vec-30 cells was 76.553%. The percentage of SEMA3B-30 cells in the S phase was significantly decreased in comparison with that in the control Vec-30 cells (4.812 and 10.500%; P<0.05; Fig. 5A).
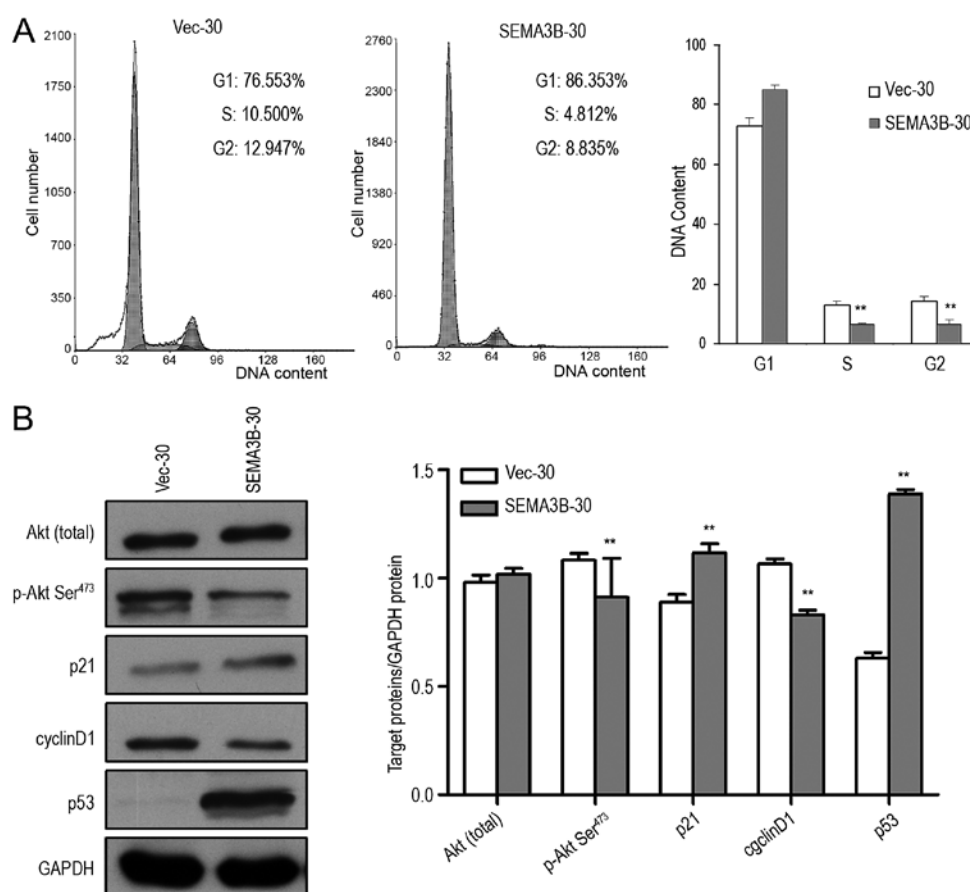
Figure 5. SEMA3B induces G1 and S cell cycle arrest. (A) Representative and summary of DNA content as detected by flow cytometry demonstrated that at the S phase, the percentage of cells was much lower in the SEMA3B-expressing (SEMA3B-30) cells than that in the control Vec-30 cells. Values are the mean ± SD of 3 independent experiments; **P<0.05. (B) Protein expression levels of p21, p53, cyclin D1, Akt (total), p-Akt (Ser473) were determined by employing western blot analysis. GAPDH served as an internal control. Quantification of the western blot results. p-Akt (Ser473), p53 and p21 expression in the SEMA3B-30 cell line was significantly higher than that noted in the Vec-30 cells, while an opposite result was found for cyclin D1. Values are the mean ± SD of 3 independent experiments; **P<0.05.

To elucidate the potential molecular mechanism of SEMA3B in cell cycle arrest, the effects of SEMA3B were examined on several key cell cycle regulators including p21, p53, cyclin D1, and Akt from the phosphatidylinositol 3-kinase/Akt pathway. Total Akt and phosphorylated Akt (Ser473) proteins were determined by western blotting with isotype-specific antibodies. According to Fig. 5B, downregulation of phosphorylated Akt at Ser473 was significantly downregulated by SEMA3B in the SEMA3B-30 cells in comparison with that in the Vec-30 cells (P<0.05), while the total Akt level was not changed (P>0.05). Furthermore, the expression of p21, p53 and cyclin D1 in the SEMA3B-30 cells was found to be increased in comparison with the Vec-30 cells (P<0.05; Fig. 5B).

Discussion

Located at the chromosomal region 3p21.3, SEMA3B is considered as a member of the class 3 Semaphorin family, a group of secreted proteins that repulse axonal extension (20). In the present study, downregulation of SEAM3B was detected in 56.7% of 60 pairs of primary ESCC cases at the mRNA level, and 66.7% of 9 ESCC cell lines, respectively. After that, the quantity of samples was expanded, so as to verify its expression at the protein level. The tissue microarray study indicated that the absence of expression of SEMA3B at the protein level was detected in 56.3% of primary ESCCs. These consistent observations indicated the downregulation of SEMA3B in ESCC. Furthermore, the relationship between gene and clinical pathological characteristics indicated that the downregulation of SEMA3B was more frequently observed in patients with lymph node metastasis (P=0.000), advanced clinical stage (P=0.001) and poor survival (P=0.017). The Kaplan-Meier analysis showed that the overall survival rate of ESCC patients decreased as SEMA3B was downregulated in the tumor tissues. The multivariate analysis indicated that downregulation of SEMA3B could be used as an independent prognostic predictor for ESCC patients. Chromosome 3 allelic loss and promoter hypermethylation may be the main cause of SEMA3B downregulation (21-26).

It is well known that SEMA3B has marked ability to induce tumor cell apoptosis and regulate cell growth in lung and breast cancer, and other solid tumors (13,14,21,27,28). In order to explore the function and mechanism of antitumorigenic properties of SEMA3B in ESCC cells, a stable SEMA3B-expressing ESCC cell line, SEMA3B-30, was established. The tumor-suppressive function of SEMA3B was investigated in our *in vitro* assay. The result indicated that SEMA3B could significantly suppress cell growth, decrease foci formation

and inhibit ESCC cell migration and invasion. Further study revealed that SEMA3B could inhibit the phosphorylation of Akt expression, indicating that the tumor-suppressive function of SEMA3B in ESCC may be correlated with the PI3K/AKT signaling transduction pathway. Known as protein kinase B, Akt is activated downstream of PI3K in response to receptor stimulation and modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression and cell growth (29-34). Phospho-Akt leads to activation of MDM-2, which targets p53 for degradation (35-38), whereas inactivation of apoptotic regulators and apoptotic factors may conversely take place. Akt activation promotes cell cycle progression and cell growth by impeding nuclear localization of p21 and p27 which are cyclin-dependent kinase inhibitors; inhibiting glycogen synthase kinase-3 (GSK-3) so as to lead to the stabilization of expression of cyclin D1; maintaining levels of the anti-apoptotic protein survivin (39-42). Our results revealed that SEMA3B was able to arrest ESCC cells at the G1/S checkpoint through inhibition of the phosphatidylinositol 3-kinase/AKT signaling transduction pathway, upregulation of p53 and p21 expression and downregulation of cyclin D1 expression.

Moreover, it has been confirmed that SEMA3B induced IL-8 secretion from tumor cell by initiating the p38-mitogen-activated protein kinase pathway. The release of IL-8 induced the recruitment of tumor-associated macrophages which may promote cancer progression and metastasis (43,44). Recent studies indicate that SEMA3B reduces invasive properties by inhibiting MMP-2, MMP-9, αvβ3 and pro-angiogenesis genes and by upregulating anti-angiogenesis genes in endometrial cancer cells (45). Previous studies indicated that TIMP3, which is an inhibitor of MMP and also a key player in tumor cell invasion, angiogenesis and cell growth processes, was found to be upregulated under SEMA restoration (46). In contrast, SEMA3B shares similar binding sites on NP-1 and NP-2 proteins with VEFG165, acting as an autocrine survival factor (28,47). SEMA3B competes with VEFG165 for binding on the tumor cell surface. Therefore, SEMA3B mediates its tumor-suppressing effects, at least in part, by blocking VEGF autocrine activity.

In summary, for the first time, the results of the present study demonstrated that downregulation of SEMA3B was frequently found in ESCC tumor specimens and predicted a worse prognosis of ESCC patients. Our results indicate that SEMA3B may be an important tumor-suppressor gene in the malignant progression of ESCC and a valuable prognostic marker for ESCC patients. Further research needs to be conducted in *in vivo* studies and the reasons for SEMA3B downregulation require further investigation.

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