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Original Article

Downgraded expression of SEMA3B indicates an unfavorable prognosis in patients of resectable hepatocellular carcinoma

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Abstract: Background: The SEMA3B protein plays an important role in suppressing tumor development as an angiogenesis inhibitor, which might be a predictable bio-marker for prognosis in hepatocellular carcinoma as our hypothesis. Methods: In this study, we have demonstrated the expression of semaphorin3B (SEMA3B) and microvascular density (MVD) in hepatocellular carcinoma (HCC) tissues. Results: We found that about 42.9% cells are SEMA3B-positive, which is significant lower compared with that of in normal liver and paraneoplastic tissues (78.6%, 85.7%, respectively). We observed that MVD in SEMA3B-positive HCC tumor tissues were lower than that of SEMA3B-negative ($P<0.05$). Our clinical data prospective study demonstrated that the lower expression of SEMA3B was closely related to the tumor nodular number, tumor size, capsulation and CLIP score ($P<0.05$) accordingly. As of rate of recurrence and metastasis, the patients in SEMA3B-positive group were significantly lower than that of the SEMA3B-negative group, also SEMA3B-positive group comes with the higher survival rate as well ($P<0.05$). Take them all together, we summarized that SEMA3B protein acts as an inhibitor though inhibiting angiogenesis, migration, and invasion during the process of human hepatocellular carcinoma. The downgraded expression level of SEMA3B closely associated with tumor progression and prognosis. Conclusion: SEMA3B could be an independent predictor of prognosis and a possible novel target of anti-angiogenic therapy for patients with HCC.

Keywords: Hepatocellular carcinoma, SEMA3B, biomarker, prognosis, invasion, migration

Introduction

Semaphorin3B (SEMA3B) is one of secreted 51 kDa protein that is processed from an 83 kDa precursor. The gene encoded SEMA3B is located in the LUCA region of chromosome 3p21.3 [1-3] and is highly methylated (~83%) in hepatocellular carcinoma (HCC) [4]. It has been reported that SEMA3B plays an important role not only in the development of the neuron axons [5], but also inducing apoptosis in lung and breast cancers [6]. VEGFs are indirect promoters of tumor growth in vivo by inducing angiogenesis that is crucial for supporting expansion of tumor mass and metastases. In addition, members of the VEGF family support tumor growth directly by acting as autocrine survival factors for those malignant cells at express VEGF or NP receptors [7-9]. VEGF and SEMA3A are antagonistic autocrine NP-1 ligands that regulate breast carcinoma cell migration [10]. The existence of common receptor(s) for both VEGF and semaphorin(s) implies that these factors might compete for the same binding sites on the cell surface. Tumor cells produce VEGF, which could act as an autocrine survival factor as well as stimulate tumor angiogenesis. Because SEMA3B exerts an anti-tumor, antiproliferative and proapoptotic effects on multiple cancer cells in vitro, we hypothesized that SEMA3B effects on tumor cell growth and viability might be due to its ability to block autocrine VEGF survival pathway by competing for the same receptor. Recent studies showed that the expression of SEMA3B is down-regulated in

many cancers including prostate and ovarian cancers [11, 12]. The down-regulation of SEMA3B mRNA expression in tumor tissues was recently confirmed by semi-quantitative PCR [4, 13-15]. However, the expression of this gene at the protein level and its clinical significance in hepatocellular cancer has not been reported.

The study protocol was approved by the ethics committee of Qilu Hospital of Shandong University. The written informed consent was obtained from all participants involved in this study. We have analyzed our recently clinical data which comes from patients who has performed curative resections, including SEMA3B expression in tumor tissues. We also demonstrated the relation of the expression of SEMA3B with clinicopathologic factors and tumor angiogenesis from our prospective study to explore the importance of SEMA3B in prognosis. In addition, we have performed migration and invasion assays in HepG2 cells treated with different siRNA silencers or SEMA3B. We found that SEMA3B has anti-motility and anti-invasive effects on tumor cells.

These results suggest that SEMA3B down-regulated expression might be an important factor to predict the prognosis of hepatocellular cancer patients and an important inhibitor to preventing migration and invasion.

Materials and methods

Clinical specimens and pathological materials

The resections of the carcinoma and tumor-adjacent (≥1 cm from the cancer tangent) tissues were collected from 56 patients with hepatocellular carcinoma. These patients (47 males and 9 females, ages 32 to 73) were hospitalized in the Department of General Surgery of Qilu Hospital, Shandong University from July 2008 to June 2009. As controls, tissues from 14 normal livers were also collected. All of these specimens were pathologically confirmed and the patients used in this study had not been treated neither radiofrequency ablation nor intervention before surgery. Each of the collected specimens was divided into two parts, one part was flash-frozen in liquid nitrogen and the other was paraffin-embedded.

Immunohistochemistry

The paraffin-embedded tissues were sectioned at a thickness of 4 μm. To study the expression of SEMA3B, the sections were stained with SEMA3B polyclonal antibody (ABCAM, UK, 1:200 in PBST) according to the manufacturer's instructions. Immunosignals were detected using streptavidin-biotin-fluorochrome (*SABC*) supplied within a kit (GeneTex, USA). To measure microvessel density (MVD), mouse anti-human CD34 monoclonal antibody (Santa, USA, 1:200 in PBST) were used and the detection procedure was similar to that used for SEMA3B. As negative controls, PBS was used instead of primary antibody.

For the purpose of quantification, semi-quantitative integration method was adopted. First, five fields on each slice were randomly selected under the magnification of 100×, and 500 cells in each field were counted under a higher magnification (200×). The appearance of yellow to brown particles in the cytoplasm was considered a positive signal according to manufacture instructions. For the purpose of statistical analyses, the staining intensity assigned as follows: no color = 0 point, yellow = 1 point, brown = 2 points and tawny = 3 points. The scores for SEMA3B-positive cells over total cells were defined as follows: ≤25% = 0 point, 25% to 50% = 1 point, 50% to 75% = 2 points and >75% = 3 points. Overall negative and positive groups were identified based on the sum of the above two scoring systems: total scores less than or equal to 3 was classified as negative group, while more than or equal to 4, positive group. To measure MVD, the five highest vascular density areas were selected under 100× light microscope, and CD34-positive cells or cell clusters in each area were counted under 200× (0.708 mm^2). The average number of the five regions was used as the MVD value. The results were confirmed by two senior pathologists independently.

Western blot

Expression of SEMA3B was also measured via Western blot. Tissues were washed with PBS, scraped, lysed, sonicated, and then centrifuged at 14,000 g for 15 min at 4°C. The protein concentrations of total cell lysates were quantified by a Bradford assay (Bio-Rad Laboratories).

Proteins were denatured by boiling in 5× loading buffer for 5 min. The sample lysates (30 μg) were loaded on 4-15% SDS polyacrylamide gels and then transferred to nitrocellulose membranes, which were blocked with 5% nonfat milk and incubated in primary antibodies to SEMA3B (Abcam, Cambridge, MA). Specific bands were visualized after incubation with secondary antibodies goat anti-rabbit IgG conjugated to HRP, by ECL, followed by exposure to film.

Semi-quantitative Real-time PCR analysis

Total RNA was extracted by using RNeasy Mini kit (Qiagen, Valencia, CA). Gene expression was quantified by real-time RT-PCR as described [16]. 0.5 μg total RNA isolated as described above was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. One microliters of cDNA was amplified in 20 μl reactions containing primers at 250 nM in iQ SYBR Green Supermix (Bio-Rad). PCR was performed for 40 cycles consisting of 95°C for 15 s and 58°C for 45 s using an iCycler iQ Real Time Detection System (Bio-Rad). Dilution curves showed that PCR efficiency was 96-100% for all primer sets used Sequences of primers used for PCR are as follow, SEMA3B sequence (forward, 5'-TTCTTTCGTGAGACGGCGGTA-3'; reverse, 5'-CCCTGGAAGATGCTGCTGGA-3'), Housekeeping gene 36B4 (forward, 5'-CGACCTGGAAGTCCAACTAC-3'; reverse, 5'-ATCTGCTGCTCTGCTTG-3'). All samples were run in triplicate on the same plate for each primer set and repeated at least 3 times in different samples. Negative controls, such as cDNA reactions without reverse transcriptase or RNA, and PCR mixtures lacking cDNA were included in each PCR reaction to detect possible contaminants. Following amplification, specificity of the reaction was confirmed by melt curve analysis. Relative quantitation was determined using the comparative CT method with data normalized to 36B4 and calibrated to the average ΔCT of untreated controls.

Cell culture

Human hepatocellular carcinoma Cell line HepG2 was purchased from the American Type Culture Collection (ATCC). Then according manufactures instructions, HepG2 (ATCC® HB-8065) was cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1% pen-strep in a 5% CO_2 incubator at 37°C. The culture medium was changed every 2-3 days.

SEMA3B siRNA suppress assays

The human SEMA3B siRNA silencer was purchased from Santa Cruz Biotechnology (Dallas, Texas). Negatively controlled siRNA (NTC-siRNA) with no homology to any known mammalian was used as a control. In vitro transfection was performed by using the siRNA Reagent System from same company. According manufacture's protocol, HCC cells were transfected by SEMA3B siRNA with final concentration of 50nmol according manufacturer's instructions. Briefly, cells were plated In a six well tissue culture plate, seeded 2×10^5 cells per well in 2 ml completed medium until the cells are 60-80% confluent. Add the siRNA duplex solution directly to the dilute Transfection using a pipette. Mix gently by pipetting the solution up and down and incubate the mixture 15-45 minutes at room temperature. Wash the cells once with 2 ml of siRNA Transfection Medium. For each well, add 0.8 ml siRNA Transfection Medium containing the siRNA Transfection Reagent mixture. Mix gently and incubate the cells 5-7 hours at 37°C in a CO_2 incubator. Then add 1 ml of 2× normal growth medium containing 2× normal serum concentration (2× normal growth medium). Incubate the cells for an additional 24 hours before change the mixed medium and replace with fresh 1x normal growth medium for other 24 hours. The transfected cells should be ready for protein test in 72 hours.

SEMA3B conditional media (CM) preparation

According Castro-Rivera reported [6], briefly, after transfected with the vector plasmid only as control, and plasmids encoding SEMA3B, the Cos7 cells were cultured in the 37°C incubator with 5% CO_2. After 48 hours, the medium was collected. Western blot was applied to determine SEMA3B present in the medium.

Migration and invasion assay

Transwell migration and invasion assays, the in vitro cell migration assay was performed as we previously described [17] in triplicate with Transwell chambers (8-um pore size; #3422, Costar, Cambridge, MA). Briefly, 5×10^4 HepG2 cells, either treated by transfection or untreated, in 100 μl plaint media (serum free) were

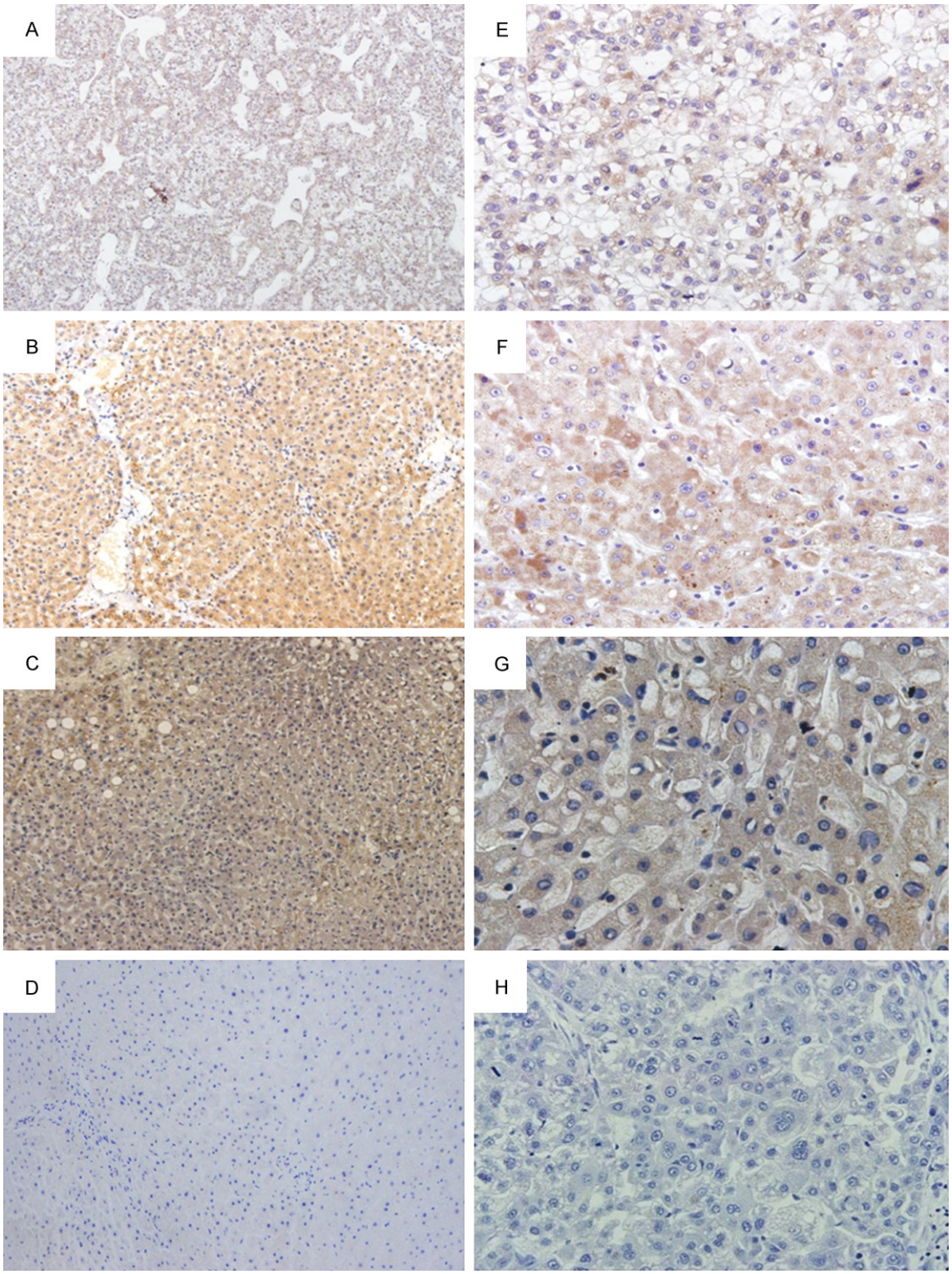

Figure 1. Expression of SEMA3B protein was down-regulated in human HCC tissues SEMA3B immunohistochemistry has been performed on human HCC tumor tissues, adjacent tissues and normal liver. Left columns show 100× optical magnification and right columns show 400×. A and E: The expression of SEMA3B in human hepatocellular carcinoma tissues (100× and 400×); B and F: The expression of SEMA3B in tumor-adjacent liver tissues (100× and 400×). C and G: The expression of SEMA3B in normal liver tissues (100× and 400×). D and H: PBS instead of primary antibody as the negative control in human HCC tumor tissues (100× and 400×).

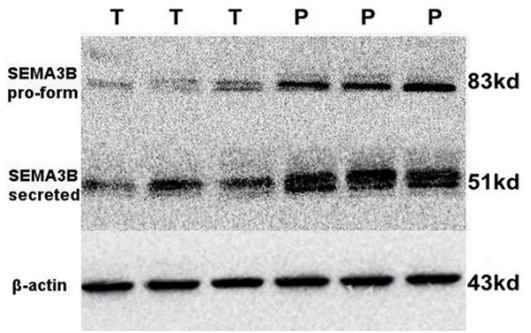

Figure 2. Western Blot: Decreased expression of SEMA3B was found in human carcinoma tissues detected by Western Blot. T: tumor tissues, P: paraneoplastic tissues.

seeded on top of the polycarbonate filters, 0.5 ml of completed growth medium (with 10% FBS) was added to the lower wells. After incubation for 12 h, filters were swabbed with a cotton swab, fixed with methanol and then stained with homatoxylin (Sigma). For the invasion assays, filters were coated with matrigel (Becton Dickinson, Franklin Lakes, NJ, USA), and 2×10^5 HepG2 cells were seeded onto the matrigel and incubated for 20 h. The migrated cells on the lower surface of the insert filter were fixed, stained with hematoxylin and mounted on glass slides. Migration was measured visually by counting using a light microscope at 40× magnification. The mean number of cells in 10 randomly chosen fields was calculated for each treatment of 4 experiments.

Statistics

For statistical comparison of counting data among the negative and positive groups, χ^2 test was used. For drawing the survival curve and comparing the differences among survival curves, Kaplan-Meier Survival Analysis and Log Rank methods were applied, respectively. All of the statistical analyses were performed with the SPSS 18.0 statistical software and a $P<0.05$ is considered to be a statistically significant difference.

Results

Expression of SEMA3B protein is down-regulated in human HCC tissues

In this study, we investigated the expression of SEMA3B protein from human hepatocellular carcinoma tissues and attempted to reveal its clinical significance. To compare the expression profiles of SEMA3B protein between normal and hepatocellular carcinoma tissues, we carried out both immunohistochemistry and Western blotting analyses. Immunostaining with the SEMA3B polyclonal antibody showed that this protein is located in the cytoplasm (**Figure 1**). The expression of this protein was significantly down-regulated ($P<0.05$) in the hepatocellular carcinoma cells (**Figure 1E**) compared to that of the cells from tumor-adjacent (**Figure 1F**) and normal liver tissues (**Figure 1G**). Statistically, the percentage of SEMA3B-positive cells in the normal liver and paraneoplastic tissues were 78.6% and 85.7% respectively, while in hepatocellular carcinoma cells only 42.9% expressed SEMA3B protein. In support of this result, our Western blotting analysis (**Figure 2**) showed that the expression level of SEMA3B protein was much lower in the cancer tissues than that in the tumor-adjacent or normal tissues (85.7% and 78.6%, respectively).

Expression of SEMA3B is negatively correlated with MVD

Microvessel density (MVD) can be measured by the number of CD34-positive endothelial cells [18, 19]. In this study, CD-34 positive cells were stained with the monoclonal anti-CD34 antibody. The number of the CD34-positive cells in the area of 0.708 mm^2 (full field area under magnification of 200) were recorded under light microscopy (**Figure 3**). To statistically study the MVD in different tissues, we defined 92 as a median value of MVD in this study. A value greater than 92 was considered as up-regulated while a number less than or equal to 92 was considered as down-regulated. It was interesting that we found that the expression profiles of MVD were in the opposite direction to those of SEMA3B protein. Specifically, the MVD value in the hepatocellular carcinoma cells was 112 ± 7.5, and this value was significantly down-regulated in the normal tissues (85.1 ± 4.4, $P<0.05$). The negative correlation between the level of SEMA3B protein and the expression of MVD may reflect that the importance of SEMA3B in the regulation of tissue vascularization.

SEMA3B is related with the clinical and pathological features of HCC

To unveil the possible role of SEMA3B in cancer diagnosis, we statistically analyzed the correlation between the expression of SEMA3B and

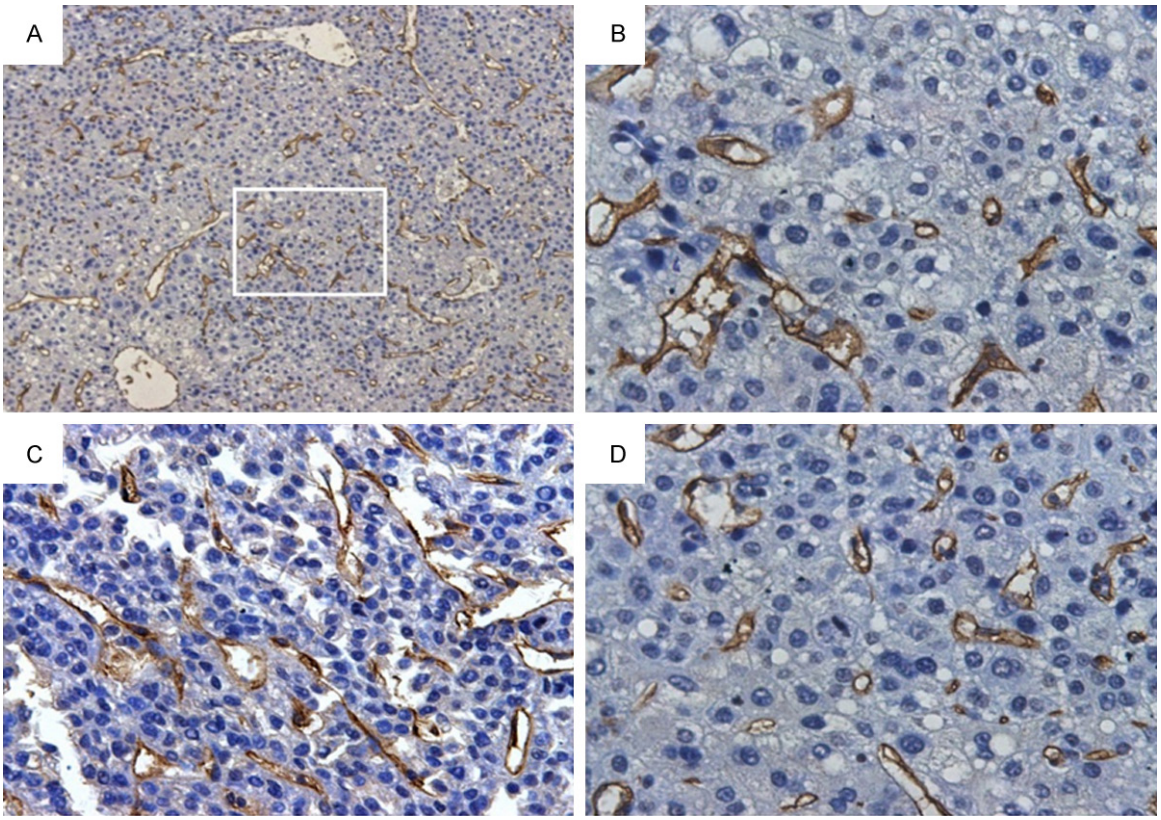

Figure 3. Expression of SEMA3B is negatively correlated with MVD (CD34+) in Human HCC tissue to show that HCC tumor tissue with SEMA3B-positive had low MVD. A: Within ×100, B: Shows magnified 400× from A frame, C and D: Show 400× CD34+ immunohistochemistry staining from different tumor tissues.

the clinical and pathological features of the 56 patients with hepatocellular carcinoma (**Table 1**). In this study, we analyzed the differences between SEMA3B-positive and SEMA3B-negative patients in the following clinical and pathological items such as gender, age, hepatitis B, AFP, liver cirrhosis, the differentiation of the tumor cells, size, with or without a complete capsule, the number of tumor nodules, CLIP score and TNM stage (**Table 1**). These analyses showed that the number of the tumor nodules in the SEMA3B-positive group was significantly higher than those in its negative group (P = 0.045). The size of tumors in the SEMA3B-positive group were statistically larger than those in the SEMA3B-negative group (P = 0.034). Furthermore, the number of the completely encapsulated tumors in SEMA3B-positive patients were much more than that in SEMA3B-negative patients (P = 0.009), while the CLIP score in SEMA3B-positive group was significantly lower than that in the SEMA3B-negative group (P = 0.013).

SEMA3B protein can be used as a clinical marker for prognosis

To study the relationship between expression of SEMA3B and prognosis, we continuously monitored the 56 patients with hepatocellular carcinoma (24 SEMA3B-Positive, 32 SEMA3B-negative, **Table 1**). Clinical follow-up studies showed that the cancer recurrence rate in the SEMA3B-positive group was significantly lower than that in the SEMA3B-negative group (P = 0.009, **Figure 4A**). Furthermore, the survival rate in SEMA3B-positive group was significantly higher than that in the SEMA3B-negative group (P = 0.020, **Figure 4B**). To assess the value of prognostic of SEMA3B, we have compared prognostic impact of SEMA3B with the other well-known prognostic markers for HCC. Univariate and multivariate analysis have been performed to test whether the expression of SEMA3B could be an independent prognostic marker. In **Table 2**, univariate analysis showed tumor capsule and SEMA3B positive were associated with tumor recurrence, multivariate

Table 1. Clinicopathologic variables associated with different expression patterns of SEMA3B

Variable	Numbers of patient		*P* value
	SEMA3B-positive	SEMA3B-negative	
Gender			0.793
Male	21	26	
Female	3	6	
Age (years)			
≤56	11	17	0.589
>56	13	15	
HBsAg status			0.735
Negative	3	2	
Positive	21	30	
AFP (ng/ml)			0.577
≤20	10	11	
>20	14	21	
Liver cirrhosis			0.543
Negative	1	4	
Positive	23	28	
Cell differentiation			0.060
Moderately/poorly	14	26	
High	10	6	
No. of tumors			0.045*
Solitary	21	19	
Multiple	3	13	
Largest tumor size (cm)			0.034*
≤5	18	15	
>5	6	17	
Tumor capsule			0.009*
Absent	8	22	
Present	16	10	
CLIP score (points)			0.013*
0-1	22	20	
≥2	2	12	
TNM stage			0.139
I	13	11	
II, III	11	21	
MVD			0.001*
≤92	18	10	
>92	6	22	

*$P<0.05$.

analysis shows that SEMA3B was an independent factor for tumor recurrence. In addition, MVD, CLIP scores and SEMA3B had relationship with cumulative survival rate of patients, but multivariate analysis shows that SEMA3B was an independent factor. Therefore, SEMA3B protein can be used as an important indicator in tumor prognosis.

The suppressive effect of SEMA3B protein on cancer cells migration and invasion ability in vitro

The SEMA3B inhibits motility of Human hepatocellular carcinoma Cell in vitro. In addition, we have performed migration and invasion assays in HepG2 cells treated with different siRNA silencers. Serum is commonly used as a chemoattractant for cell migration and invasion assays because serum contains many cytokines and growth factors. By seeding cells in serum-free media, a chemoattractant gradient is established between the cells in the upper chamber and the 10% serum in the lower chamber, which gives the cells an incentive to invade through the gel layer. The completed growth media contained 10% FBS which to effectively induce migration of HepG2 cells, A role for SEMA3B protein in this process was investigated by either inhibited by siRNA silencer or enhanced by SEMA3B CM. As illustrated in **Figure 5**, the ability of the cells to migrate into the lower wells was inhibited in SEMA3B enhanced CM significantly ($P<0.05$) and upgraded in SEMA3B siRNA silencer treatment ($P<0.01$).

It is noticeable that HepG2 cells' migration increased about 35% by in siRNA silencing of SEMA3B expression while decreased about 30% in SEMA3B conditional medium which enhanced SEMA3B expression. The invasion ability assay was the same pattern as migration analysis.

Discussion

SEMA3B, encoded at 3p21.3, is recently recognized as a candidate tumor suppressor gene [4, 5]. Evidence in support of this finding includes the following: frequent allele loss at the SEMA3B 3p21.3 locus; frequent loss of SEMA3B expression in multi-cancer secondary to tumor-acquired promoter methylation; occasional somatically acquired tumor SEMA3B mutations; and inhibition of tumor cell growth in vitro and in vivo coupled with dramatic ability to induce tumor cell apoptosis after transfection or exposure to SEMA3B protein [1, 4, 5]. SEMA3B, as a tumor suppressor gene, is a secreted protein and processed from its precursor. This protein is encoded by SEMA3B gene and belongs to the semaphorin/collapsin family [19, 20]. SEMA3B from the family play critical roles in the development of neuronal

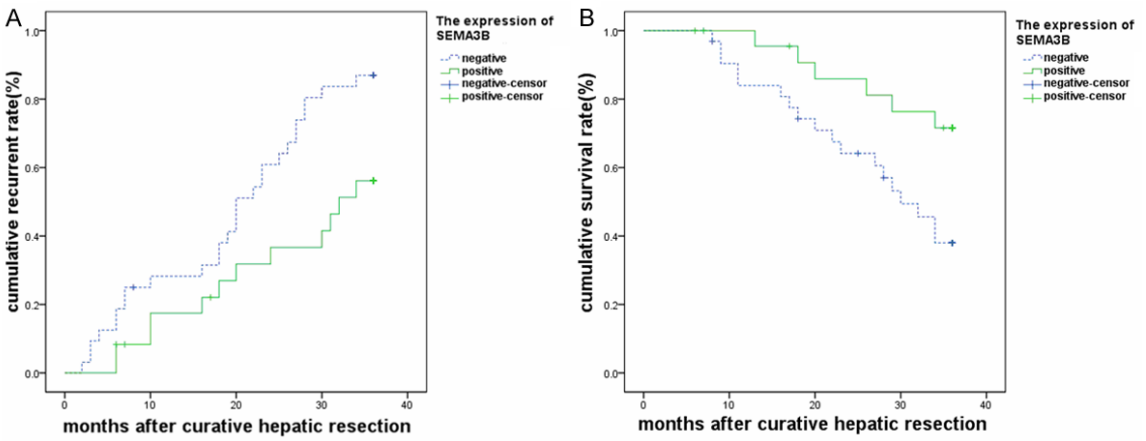

Figure 4. Prognosis A: The recurrence rate of positive group was significantly lower than the negative group (P = 0.009). B: The survival rate of the positive group was significantly higher than the negative group (P = 0.020).

axon and possibly act as tumor suppressors by inducing apoptosis [20, 21].

In this study, we have investigated the expression profile of SEMA3B protein in the tissues, from both HCC and normal tissues. In the HCC tissues, where SEMA3B was down-graded, MVD value was significantly increased compared with that in the normal tissues. The negative correlation between the level of SEMA3B protein and the expression of MVD may reflect that the importance of SEMA3B in the regulation of tissue vascularization. The results suggest that SEMA3B acts might be through inhibiting angiogenesis to mediate its tumor suppressor effects. The expression detection in human tumor demonstrated that, for the first time, SEMA3B protein is significantly down-regulated in HCC tissues and its low expression is possibly the result of high value of MVD in this cancer tissue. Specifically, the SEMA3B-positive cells in HCC were dramatically less than that in normal liver cells (78.6%) and tumor-adjacent tissues. These results are consistent with previous data reported from the studies at mRNA level, which showed that transcription of SEMA3B gene is down–regulated in hepatocellular carcinoma tissues [4, 22, 23]. The low expression of SEMA3B in the HCC tissues can come from many pathways. We assumed that SEMA3B protein have had consumed on procedure of tumor formation, like competitive inhibited by VEGF on occupied with cells receptor in result of the high value of MVD on this cancer tissue. The other possibility is methylation of the promoter of its gene since this promoter was hypermethylated (47%) in non-small cell lung cancer [4, 13, 24]. Other factors may also contribute to this down-regulation. For example, p53, a direct apoptosis inducer, can directly regulate the expression of SEMA3B gene [25].

The negative correlation of the expression of SEMA3B protein with MVD further confirmed that SEMA3B may play an important role in angiogenesis since MVD is an important indicator for evaluating both internal local microcirculation in a tumor and the stage of its angiogenesis [26, 27]. It has been shown that SEMA3B is directly involved in the formation of blood vessels [27]. Tumors with high value of MVD resulted in poor prognosis and shorten survival time, and they are also very invasive and metastatic [28]. In 1990s, Plate found that VEGF is a potential tumor angiogenesis factor in human gliomas [29]. Carmeliet's recently study [30] showed that VEGF plays the key mediator of angiogenesis in tumor develop process. The other literatures observed that NP-1 supports a VEGF signaling pathway that is critical for breast carcinoma cell survival [9] and that VEGF and SEMA3A are antagonistic NP-1 ligands that regulate breast carcinoma cell migration [10]. Also, SEMA3A induces apoptosis [31, 32] competes with VEGF-A binding on porcine aortic endothelial, medulloblastoma, human embryonic kidney 293 cells and affects cell survival and migration [33-35]. Recent research also shows SEMA3F (whose gene is located 70 kb telomeric of SEMA3B in the same 3p21.3 region) and VEGF as having opposing effects on cell attachment and motility in breast cancer lines MCF-7 and C100 [36]. SEMA3F inhibited lamellipodia formation, membrane ruffling, and

Table 2. Univariate and multivariate analysis of SEMA3B expression compared with multiple HCC factors on tumor recurrence and survival rate

Variable	No. of patients	Recurrence rate		Survival rate	
		Univariate analysis (*p* value)	Multivariate analysis (*p* value)	Univariate analysis (*p* value)	Multivariate analysis (*p* value)
Gender					
Male	47	0.250		0.796	
Female	9				
Age (years)					
≤56	28	0.240		0.948	
>56	28				
HBsAg status					
Negative	5	0.275		0.187	
Positive	51				
AFP (ng/ml)					
≤20	21	0.214		0.611	
>20	35				
Liver cirrhosis					
Negative	5	0.741		0.275	
Positive	51				
Cell differentiation					
Moderately/poorly	40	0.051		0.629	
High	16				
No. of tumors					
Solitary	40	0.874		0.939	
Multiple	16				
Largest tumor size					
≤5 cm	33	0.166		0.275	
>5 cm	23				
Tumor capsule					
Absent	30	0.044*	0.227	0.640	
Present	26				
CLIP score					
0-1	42	0.331		0.035*	0.353
≥2	14				
TNM stage					
I	24	0.350		0.902	
II/III	32				
MVD					
≤92	28	0.380		0.040*	0.178
>92	28				
SEMA3B					
Negative	32	0.009*	0.013*	0.020*	0.027*
Positive	24				

*$P<0.05$.

cell-cell contacts through interaction with NP-1 [36]. There are bunch of recent reports demonstrated that SEMA3B could inhibit proliferation [37, 38]. Forced overexpression of SEMA3B in either lung or ovarian cancer cells can induce cell necrosis and inhibit tumor cell growth [24]. SEMA3B family proteins can competitively inhibit the function of VEGF in promoting tumor angiogenesis [6] since they share the same trans-membrane receptors of NP-1 and NP-2 [39]. The involvement of SEMA3B in the occurrence and growth of HCC could be through many pathways.

The findings reported in this study show that the single nodules in SEMA3B-positive group were significantly higher than the multiple nodules could be due to nuclear matrix proteins (NMPs). Clinical correlation analyses showed that expression level of SEMA3B is closely related to the formation of capsule, possibly via inhibitory effect of SEMA3B on the formation of the blood vessels [40]. SEMA3B can inhibit the expression and activity of MMP-2 and MMP-9 [41, 42]. Taken together, these data suggest that SEMA3B is likely a tumor suppressor gene and may be used as a clinical marker for analyzing tumor development.

Our clinical data also demonstrated that the SEMA3B was negatively correlated with TNM stage and the CLIP score ($P<0.05$). CLIP score was proposed in 1998 by the Italian Liver Cancer Group and plays a good

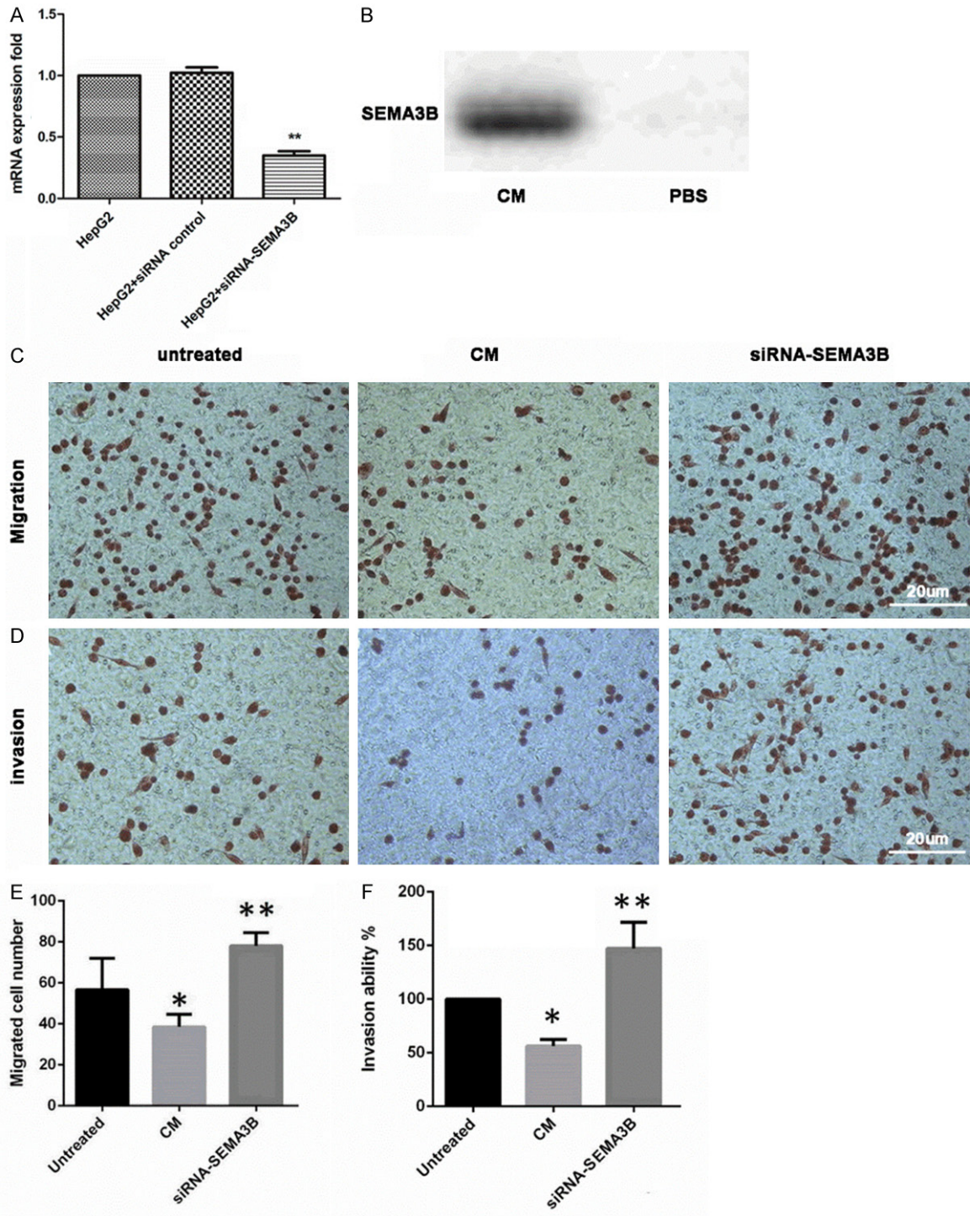

Figure 5. The suppressive effect of SEMA3B protein on cancer cells migration and invasion ability in vitro. The CM means conditional media collected from cos-7 cells which SEMA3B has been enhanced. A: Expression of SEMA3B mRNA. B: Expression of protein SEMA3B in CM. Transwell migration and invasion assays were performed on Hep-G2 cells. C-F: The migration assay demonstrated the inhibitory effects of SEMA3B protein on invasive cell migration. Hep-G2 cells cultures were plated on Transwells containing 8 um pore size, either untreated control, or treated by CM, siRNA knockdown of SEMA3B. Data expressed as mean ± SD (n = 5), *P<0.05 and **P<0.01.

role in assessing the prognosis of the patients underwent surgery [43]. Cases follow-up in this study showed that the relapse and migrating rate in the SEMA3B positive group is much

lower than that in the SEMA3B negative group, while the cumulative survival rate is significantly higher than the SEMA3B negative group. Therefore, SEMA3B can be used as a valuable indicator to measure the prognosis of the patients with HCC.

SEMA3B participates in the regulation of glioma cells invasion [44]. It is noticeable that HepG2 cells' migration increased by in knockdown of SEMA3B expression while decreased in SEMA3B CM which enhanced SEMA3B expression. The invasion ability assay was the same pattern as migration analysis. These data strongly suggest that SEMA3B has anti-motility and anti-invasive effects on tumor cells.

In summary, we have conduct the systematically investigations the prognostic value of expression of SEMA3B protein in HCC patients. Since SEMA3B is a secreted protein, it can be detected in the peripheral blood serum, which can be used for either the diagnosis of HCC or prediction of the prognosis of patients with HCC. Although the mechanism of the SEMA3B in cancer development is not fully understood so far, the results presented in this study provide the possibility that SEMA3B can be a potential clinical marker for predicting clinical diagnosis and prognosis of HCC.

Disclosure of conflict of interest

None.

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