Novel role of semaphorin 3A in the growth and progression of hepatocellular carcinoma

XUEDONG LI[1*], QING CHEN[1*], DAN YIN[1,2], SHIMING SHI[1], LEI YU[1], SHAOLAI ZHOU[1], ERBAO CHEN[1], ZHENGJUN ZHOU[1], YINGHONG SHI[1], JIA FAN[1-3], JIAN ZHOU[1-3] and ZHI DAI[1,3]

[1]Liver Cancer Institute, Zhongshan Hospital, [2]Institute of Biomedical Sciences, and [3]State Key Laboratory of Genetic Engineering, Fudan University, Shanghai 200032, P.R. China

Received November 17, 2016; Accepted December 20, 2016

DOI: 10.3892/or.2017.5616

Abstract. Semaphorin 3A (SEMA3A), a secretory protein, is a founding member of the semaphorin family and functions in both the biological behavior of tumor cells and the modulation of tumor-associated macrophages. However, the role of SEMA3A in hepatocellular carcinoma (HCC) is still not well established. In the present study, we investigated the expression levels of SEMA3A in 80 HCC tissues and cell lines, using RT-qPCR, western blotting and immunohistochemistry. Expression profile analysis revealed that SEMA3A was significantly overexpressed in human HCC patients and positively correlated with the metastatic potential of HCC cells. Lentiviral transfection into PLC/PRF/5 and HCCLM3 cells was performed to stably upregulate and downregulate the expression of SEMA3A in HCC cells. Cell Counting Kit-8 (CCK-8), wound-healing and invasion assays revealed that SEMA3A promoted the proliferation and migration of HCC cells *in vitro*. Proteome profiler antibody microarray analysis revealed that overexpression of SEMA3A in HCC cells induced a significant increase in the expression levels of gelsolin-like capping protein (CapG), galectin-3, enolase 2 and epithelial cell adhesion molecule (EpCAM). Furthermore, the upregulation of SEMA3A in HCC cells promoted tumor growth and progression in an HCC mouse model. These results indicate that SEMA3A enhances CapG, galectin-3, enolase 2 and EpCAM expression to promote HCC progression and is a potential therapeutic target for HCC.

Correspondence to: Dr Zhi Dai or Dr Jian Zhou, Liver Cancer Institute, Zhongshan Hospital, Fudan University, 136 Yixueyuan Road, Shanghai 200032, P.R. China
E-mail: doctordaizhi@163.com
E-mail: doctorzhoujian99@163.com

*Contributed equally

Key words: semaphorin 3A, hepatocellular carcinoma, tumor growth, gelsolin-like capping protein, galectin-3, enolase2, epithelial cell adhesion molecule

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with a high mortality rate and ominous prognosis, and is a serious public health threat (1,2). The high rates of recurrence and metastasis after hepatectomy are major causes of death in patients with HCC. However, the exact mechanisms of recurrence and metastasis of HCC are still poorly understood (3). Therefore, identifying novel molecular markers in HCC and clarifying the molecular mechanisms responsible for the recurrence and metastasis may be helpful for the treatment of HCC and to improve the survival and prognosis of HCC patients.

Semaphorin 3A (SEMA3A), a secretory protein, is a founding member of the semaphorin family and has a Sema domain, a PSI domain and an immunoglobulin-like domain (4). SEMA3A functions in autocrine and paracrine manners (5). Numerous studies have found that SEMA3A plays an important role in the development and in related disorders of the nervous, immune, cardiovascular and skeletal systems (6-8). Recent studies also found that SEMA3A regulates the biological behavior of tumor cells directly or affected them indirectly, by influencing the tumor microenvironment (9,10). SEMA3A is overexpressed in glioblastoma, pancreatic, lung and prostate cancer (11-14). Its increased expression is positively correlated with advanced malignant grade, cell migration, metastatic potential and poor prognosis (12,15). Furthermore, SEMA3A directly decreases focal adhesions, induces cancer cell invasion *in vitro*, and modulates tumor-associated macrophages (15,16). However, the role of SEMA3A in HCC is not well established.

In the present study, we reported that SEMA3A serves as a novel hallmark for HCC. We demonstrated that SEMA3A significantly promotes HCC progression. Notably, our findings indicate that SEMA3A promotes the proliferation and invasion of HCC by upregulating the expression of gelsolin-like capping protein (CapG), galectin-3, enolase 2 and epithelial cell adhesion molecule (EpCAM). Ultimately, these findings contribute to our current understanding of HCC progression.

Materials and methods

Patient and tissue specimens. Specimens of HCC and corresponding adjacent non-tumorous liver tissues (ANLTs) were

obtained from 80 HCC patients undergoing curative hepatic resection at the Department of Hepatic Surgery, Zhongshan Hospital of Fudan University from January 2010 to December 2010. The diagnosis for each patient was confirmed histopathologically. These tissue specimens were freshly collected for real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR) and western blot detection. Prior informed consent was obtained, and the study protocol was approved by the Ethics Committee of Zhongshan Hospital.

RNA isolation and RT-qPCR. Total RNA was extracted from cell lines and frozen tissue specimens using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The mRNA expression in HCC cell lines, 80 cases of HCC tissues and corresponding ANLTs was assessed by RT-qPCR using the Power SYBR-Green PCR Master Mix kit on an ABI 7900HT Fast Real-Time PCR instrument (both from Applied Biosystems). The primers used were as follows: SEMA3A forward, 5'-GCA AACTATCAGAATGGGAAGA-3' and reverse, 5'-GCTCCAA CATACAGCCTACTCC-3'; GAPDH forward, 5'-AGGTCGGA GTCAACGGATTTG-3' and reverse, 5'-GTGATGGCATGGA CTGTGGT-3'. The relative mRNA expression level of SEMA3A was normalized to GAPDH. The results were analyzed using the $2^{-\Delta Ct}$ method, and the formula was: $\Delta Ct = Ct_{SEMA3A} - Ct_{GAPDH}$. All of the experiments were performed in triplicate.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections were stained for SEMA3A using the avidin-biotin-peroxidase complex system (Gene Tech, Shanghai, China) according to the manufacturer's instructions. Briefly, after rehydration and microwave antigen retrieval, rabbit anti-human polyclonal antibodies against SEMA3A (1:200 dilution; Abcam, Cambridge, MA, USA) were applied to the slides, and incubated at 4˚C overnight. Then, the slides were incubated with secondary antibody (Gene Tech) at 37˚C for half an hour. Staining was performed with diaminobenzidine (DAB), and Mayer's hematoxylin was used for counterstaining. Negative control slides were probed with bovine serum albumin (BSA) under the same experimental conditions.

Western blotting. Total cell lysates were generated, and total protein was extracted and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were washed and blocked, and then incubated with rabbit anti-human polyclonal antibody against SEMA3A (1:800 dilution), rabbit anti-human polyclonal antibody against CapG (1:1,000), rabbit anti-human polyclonal antibody against galectin-3 (1:1,000), rabbit anti-human polyclonal antibody against enolase 2 (1:800), or rabbit anti-human polyclonal antibody against EpCAM (1:1,000), and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:3,000) (all from Abcam). GAPDH was detected using rabbit anti-human monoclonal antibody against GAPDH (1:2,000; Abcam) as a loading control. All antibody bindings were detected by an enhanced chemiluminescence (ECL) assay (Millipore). The bands were analyzed using the Gel-Pro Analyzer 32 software.

Cell lines and cell culture. Five human HCC cell lines (PLC/PRF/5, HepG2, MHCC97-L, MHCC97-H and HCCLM3) with different invasive and metastatic potentials were used in the present study. PLC/PRF/5 and HepG2 cell lines (human HCC cell lines with low metastatic potential) were purchased from the Chinese Academy of Sciences (Shanghai, China). HCC cell lines with stepwise metastatic potential (MHCC97-L, MHCC97-H and HCCLM3, which have the same genetic background, but different lung metastatic potentials) were established at our institute (17,18). These cell lines were routinely maintained and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA) in a humidified incubator containing 5% CO_2 at 37˚C.

Cell transfection and clone selection. The pSV40-GFP-SEMA3A and pGCSIL-GFP-shRNA-SEMA3A lentiviral vectors were purchased from the Shanghai GeneChem Co. (Shanghai, China), and the target shRNA sequence was as follows: 5'-TGTTTACAATGGTGGGAAA-3'. The pSV40-GFP-SEMA3A lentivirus was transfected into PLC/PRF/5 cells with a low metastatic potential, and the pGCSIL-GFP-shRNA-SEMA3A lentivirus was transfected into the HCC cells with a high metastatic potential (HCCLM3). The pSV40-GFP and pGCSIL-GFP lentiviral vectors were used as negative controls. Then, stably transfected clones were validated by RT-qPCR and western blotting for SEMA3A.

Cell proliferation, wound-healing and Matrigel invasion assays. For the cell proliferation assay, cells (20,000 cells/ml) were dispensed in 100 μl aliquots into a 96-well plate. At the indicated time-points, 10 μl of Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to each well, and the plate was incubated for an additional 2 h. The absorbance at 450 nm was assessed to determine the number of viable cells in each well.

For the wound-healing assay, cells were seeded into 3.5 cm dishes and cultured for 1-2 days. When the cells were 100% confluent and formed a monolayer, the medium was changed to serum starve the cells. After serum starvation, the cell monolayer was wounded with a plastic pipette tip. The remaining cells were carefully washed with culture medium to remove the cell debris, and cultured in an incubator at 37˚C for an additional 48 h. At the indicated time-points, migrating cells at the wound front were photographed using an inverted microscope (Olympus, Tokyo, Japan). The percentage of the wound closure at each time-point compared with time zero was assessed using Image-Pro Plus v6.2 software.

Matrigel invasion assays were performed using 24-well Transwell plates (8-μm pore size; Corning, Corning, NY, USA). First, ~$5x10^4$ cells were suspended in 200 μl of DMEM with 1% (v/v) FBS, and were placed into the upper chamber of the insert pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and 500 μl of DMEM with 10% (v/v) FBS was added to the lower chamber. After a 48-h incubation,

the Matrigel and the cells remaining in the upper chamber were removed with cotton swabs. Then, the cells that passed through and adhered onto the lower surface of the membrane were fixed in 4% paraformaldehyde for 15 min, and stained with crystal violet. The cells were counted and photographed in at least 5 random microscopic fields (magnification, x200). All of the experiments were performed in triplicate.

Proteome profiler antibody array assay. The Proteome Profiler™ Human XL Oncology Array (ARY026) was purchased from the R&D Systems, Inc. (Minneapolis, MN, USA). Proteome profiler antibody microarray analysis was performed according to the protocol provided. Briefly, relative expression levels of human cancer-related proteins in samples (total proteins extracted from PLC/PRF/5-SEMA3A or PLC/PRF/5-control cell lysates) were determined using the following procedure: each array membrane was placed in a separate well of the 4-well Multi-Dish with 2 ml of array buffer 6, and incubated for 1 h on a rocking platform shaker; then, the array buffer 6 in each well of the 4-well Multi-Dish was replaced with prepared samples (0.5 ml total protein + 0.5 ml array buffer 4 + 0.5 ml array buffer 6), and the array membrane was incubated overnight at 4˚C. The following day, each membrane was washed 3 times with 1X washing buffer, and then incubated in 1.5 ml of array buffer 4/6 with 30 μl of a detection antibody cocktail for 1 h. Subsequently, each membrane was washed 3 times again, and then incubated in 2 ml of diluted streptavidin-HRP for 30 min. The membranes were washed an additional 3 times, and then evenly covered with 1 ml of the prepared Chemi Reagent Mix and incubated for 1 min. Ultimately, the array membranes were exposed in the Tanon-5200 Chemiluminescent Imaging System (Tanon, Shanghai, China), and analyzed with the Gel-Pro Analyzer 32 software. The fluorescence intensity of each spot on the array membranes was quantified, and the mean value was calculated.

HCC metastatic mouse model. Approximately $5x10^6$ cells of the different groups (HCCLM3-shRNA-SEMA3A, HCCLM3-control, PLC/PRF/5-SEMA3A and PLC/PRF/5-control) were suspended in 100 μl of DMEM, and then subcutaneously injected into the upper left flank regions of nude mice (BALB/c-nu/nu, male, 4 weeks old; 3 in each group). After ~4 weeks, when the subcutaneous tumor size reached ~1 cm in diameter, the subcutaneous tumor tissues were removed, minced into small pieces of equal volume (~2x2x2 mm^3), and then implanted into the livers of other nude mice (BALB/c-nu/nu, male, 4 weeks old; 6 in each group). All of the mice were monitored on alternate days and sacrificed 6 weeks later. All of the livers of the mice were removed, and the volumes of liver tumors were calculated as follows: V (cm^3) = $3ab^2/4$ (a and b, respectively represent the largest and smallest tumor diameters measured by vernier caliper) (19). All of the lungs were also removed, fixed in 4% paraformaldehyde for 2-3 days, embedded in paraffin, serially sectioned and stained with hematoxylin and eosin. The total number of lung metastases was examined and counted under a microscope, and the lung metastatic lesions were classified into 4 grades (grade I, ≤20; grade II, 20-50 tumor cells; grade III, 50-100; and grade IV, >100 tumor cells) on the basis of the number of tumor cells present at the maximal section for each metastatic lesion as previously described (20). All of the mice used in the present study, were purchased from the Chinese Academy of Sciences, and housed under specific pathogen-free (SPF) conditions. All of the mice received humane care and were manipulated according to the protocols (approved by the Medical Experimental Animal Care Commission) and the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' (prepared by the National Academy of Sciences and published by the National Institutes of Health, NIH publication 86-23 revised 1985).

Statistical analysis. All of the data were analyzed using IBM SPSS Statistics for Windows (version 19.0.0). Values are expressed as the mean ± SD. Quantitative data were compared between groups using Student's t-test. Categorical data were analyzed using the Chi-square or Fisher's exact tests. All of the statistical analyses used a two-sided test, with $P<0.05$ as statistically significant.

Results

SEMA3A is upregulated in human HCC tissues. Both the mRNA and protein expression levels of SEMA3A were detected by RT-qPCR and immunohistochemistry in 80 cases of HCC and the corresponding ANLTs. RT-qPCR revealed that SEMA3A was significantly overexpressed in the HCC tissues when compared to the ANLTs ($P<0.01$) (Fig. 1A). The results were confirmed by immunohistochemical analyses on the aforementioned 80 samples (Fig. 1B). These data demonstrated that SEMA3A is highly expressed in HCC.

The expression level of SEMA3A is associated with the invasive and metastatic potential of HCC cell lines. The expression levels of SEMA3A in 5 established HCC cell lines were assessed by RT-qPCR and western blotting. The results revealed that both the mRNA and protein expression levels of SEMA3A were significantly higher in the highly invasive and metastatic HCC cell lines (MHCC97-L, MHC97-H and HCCLM3) than in the HCC cell lines with low invasive and metastatic potential (PLC/PRF/5 and HepG2; $P<0.05$) (Fig. 1C and D). These findings indicate that the expression of SEMA3A is positively correlated with the invasive and metastatic potential of HCC cells.

SEMA3A promotes proliferation, migration and invasion of HCC cells in vitro. To further explore the biological functions of SEMA3A in HCC, we used the lentiviral transfection approach to stably upregulate or downregulate the expression of SEMA3A in the transfected HCC cell lines (Fig. 2A-D). SEMA3A expression was ascertained by RT-qPCR and western blotting (Fig. 2E and F). Downregulation of SEMA3A by shRNA in HCCLM3 cells significantly suppressed cell proliferation after 24 h ($P<0.05$) (Fig. 3A). In contrast, cell proliferation of PLC/PRF/5-SEMA3A cells was significantly higher than that of the PLC/PRF/5-control cells ($P<0.05$) (Fig. 3B).

Furthermore, the migration and invasion abilities of the HCCLM3-shRNA-SEMA3A and PLC/PRF/5-SEMA3A cells, and the corresponding control cell lines were investigated using wound-healing and invasion assays. In the wound-healing

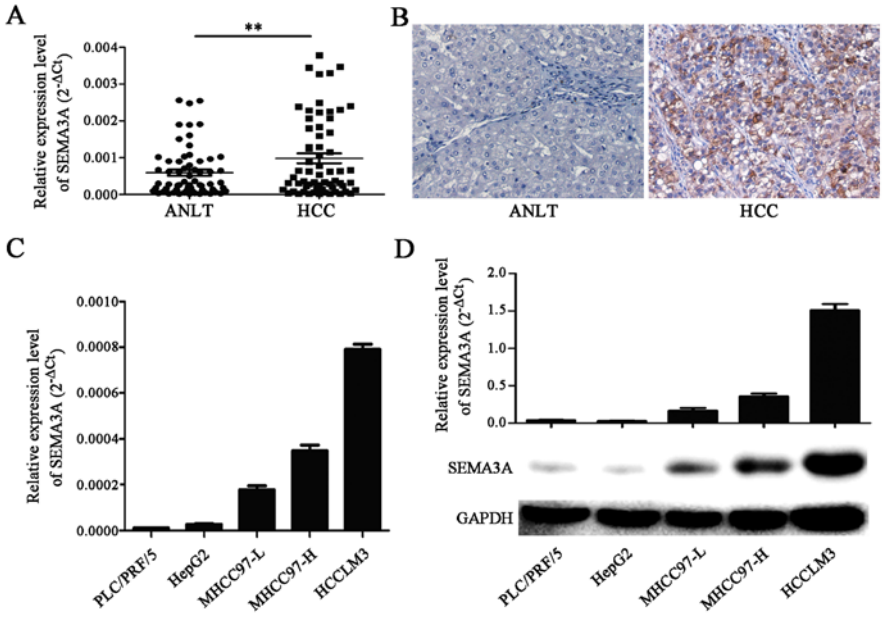

Figure 1. SEMA3A expression in HCC tissues and cell lines. (A) RT-qPCR revealed that the expression level of SEMA3A mRNA in HCC tissues was increased when compared with that in the ANLTs (n=80; **P<0.01). (B) Immunohistochemical analysis revealed that the SEMA3A protein was overexpressed in the HCC tissues (magnification, x400). (C) RT-qPCR revealed a significantly increased expression of SEMA3A mRNA in the highly invasive HCC cell lines (MHCC97-L, MHCC97-H and HCCLM3) compared with the PLC/PRF/5 and HepG2 cells (P<0.05). (D) Western blotting revealed a significant increase of the SEMA3A protein in the highly invasive HCC cell lines, MHCC97-L, MHCC97-H and HCCLM3 (P<0.05). SEMA3A, semaphorin 3A; HCC, hepatocellular carcinoma; ANLTs, adjacent non-tumorous liver tissues.

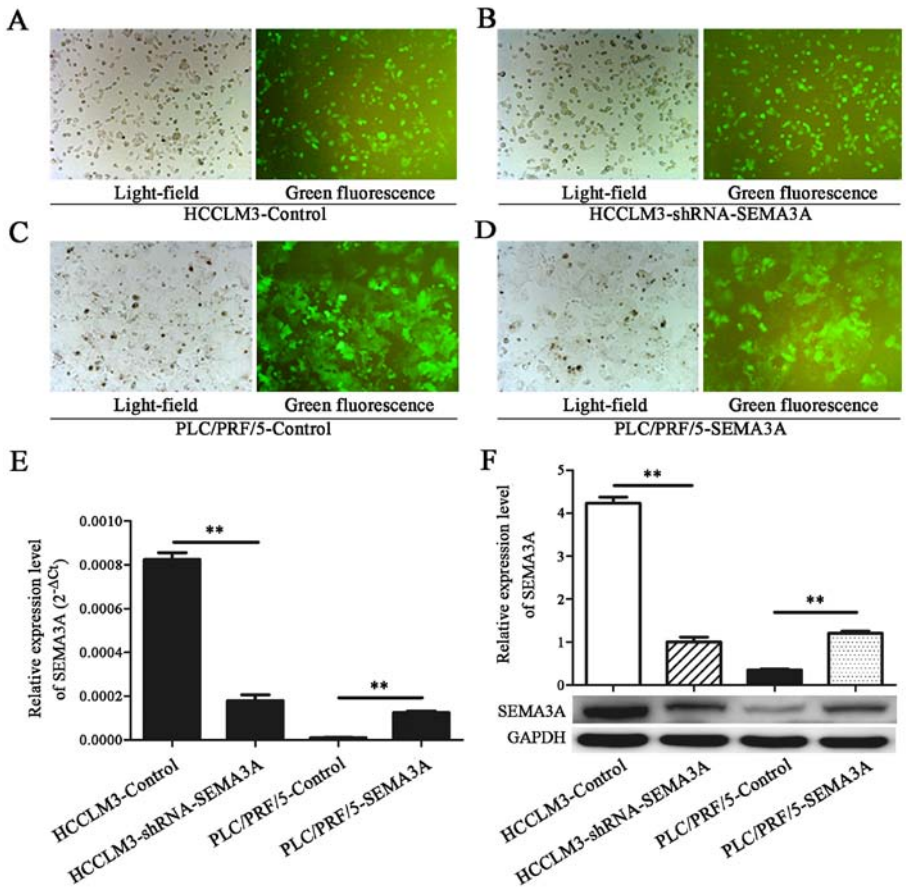

Figure 2. Stable upregulation or downregulation of the expression of SEMA3A in transfected HCC cell lines. (A-D) Fluorescence microscopic analysis revealed that the lentiviral transfection efficiencies in the HCCLM3-control, HCCLM3-shRNA-SEMA3A, PLC/PRF/5-control and PLC/PRF/5-SEMA3A cells exceeded 95% (magnification, x200). (E) RT-qPCR revealed that SEMA3A mRNA was significantly downregulated in the HCCLM3-shRNA-SEMA3A cells and significantly upregulated in the PLC/PRF/5-SEMA3A cells (**P<0.01). (F) Western blotting confirmed that the SEMA3A protein was significantly decreased in the HCCLM3-shRNA-SEMA3A cells and significantly increased in the PLC/PRF/5-SEMA3A cells (**P<0.01). SEMA3A, semaphorin 3A; HCC, hepatocellular carcinoma.

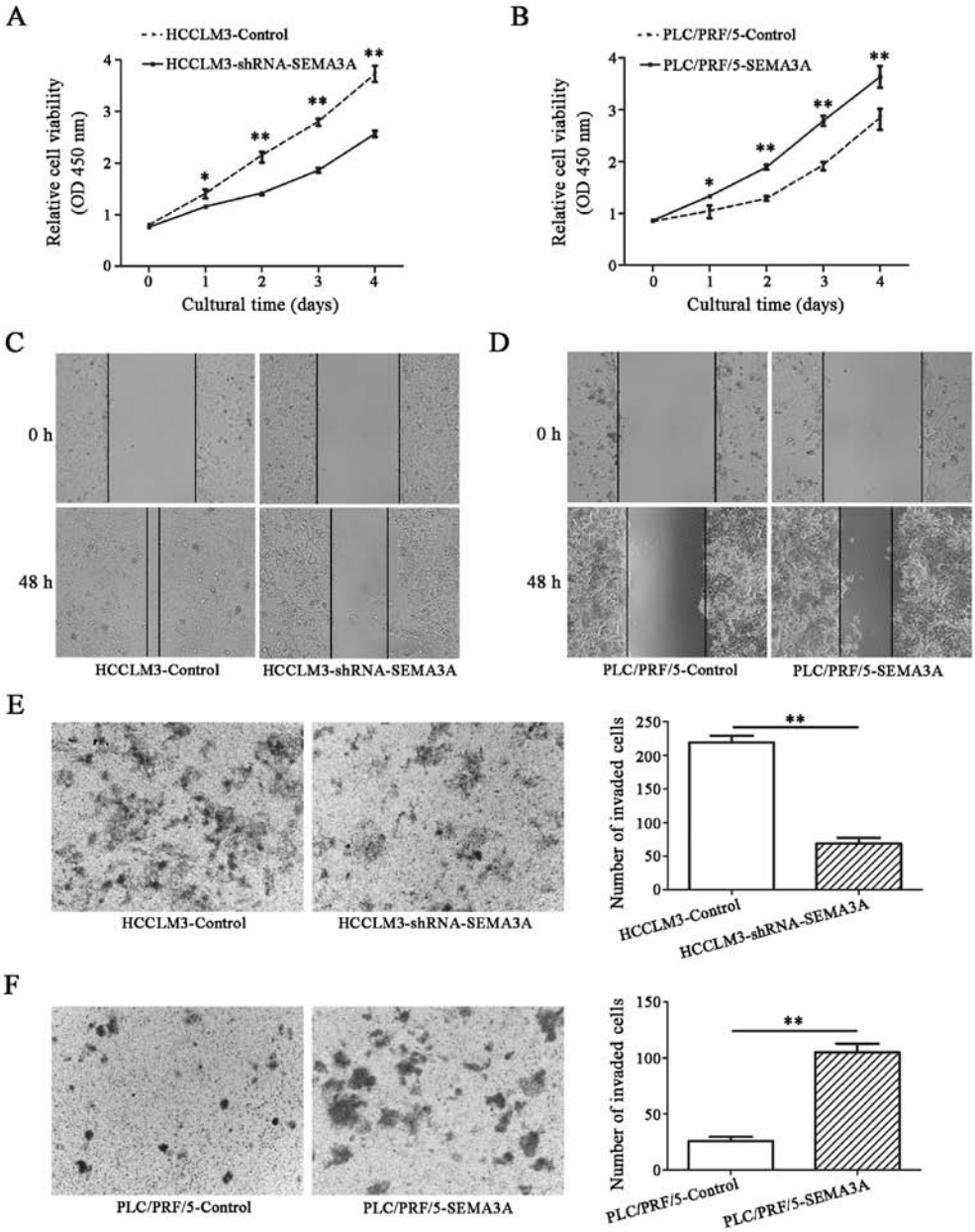

Figure 3. Effect of SEMA3A on the biological behavior of HCC cells. (A) Cell proliferation was detected by CCK-8 assay. The results revealed that cell proliferation of HCCLM3-shRNA-SEMA3A was significantly suppressed (*P<0.05; **P<0.01). (B) CCK-8 assay revealed that cell proliferation was significantly enhanced in the PLC/PRF/5-SEMA3A cells (*P<0.05; **P<0.01). (C) Wound-healing assay was performed to investigate cell migration ability. The results showed that the wound closure of the HCCLM3-shRNA-SEMA3A cells was significantly slower than that of the HCCLM3-control cells (**P<0.01). (D) Wound-healing assay revealed that the wound closure of PLC/PRF/5-SEMA3A cells was significantly faster than that of the PLC/PRF/5-control cells (**P<0.01). (E) In the Matrigel invasion assay, the results revealed that the invasion of HCCLM3-shRNA-SEMA3A cells was significantly decreased (**P<0.01). (F) Invasion assay revealed a significant increase in the number of invasive PLC/PRF/5-SEMA3A cells (**P<0.01). (C-F) Magnification, x200. SEMA3A, semaphorin 3A; HCC, hepatocellular carcinoma.

assays, microscopic examination at 48 h post-wounding revealed a significant delay in the wound closure rate of the HCCLM3-shRNA-SEMA3A cells compared with the HCCLM3-control cells (29.4±3.1 vs. 91.5±4.5%, respectively; P<0.01) (Fig. 3C), whereas PLC/PRF/5-SEMA3A cells had a significant increase in the wound closure rate compared with the control cell line (43.1±3.8 vs. 19.9±2.9%, respectively; P<0.01) (Fig. 3D). In addition, invasion assays revealed that the number of invasive HCCLM3-shRNA-SEMA3A cells was significantly less than that of the invasive HCCLM3-control cells (69.0±8.3 vs. 219.3±9.8, respectively; P<0.01) (Fig. 3E) while PLC/PRF/5-SEMA3A cells were significantly more invasive than the PLC/PRF/5-control cells (105.0±7.6 vs. 25.7±4.1, respectively; P<0.01) (Fig. 3F).

SEMA3A upregulates the expression of CapG, galectin-3, enolase 2 and EpCAM in HCC cells. To explore which signaling pathways contributed to HCC proliferation and invasion induced by SEMA3A, PLC/PRF/5-SEMA3A and PLC/PRF/5-control cells were investigated for the activation status of multiple pathways, which was determined by Proteome Profiler Human XL Oncology Array assays. Notably, we found that overexpression of SEMA3A in PLC/PRF/5 cells caused a significant increase in the expression levels of CapG,

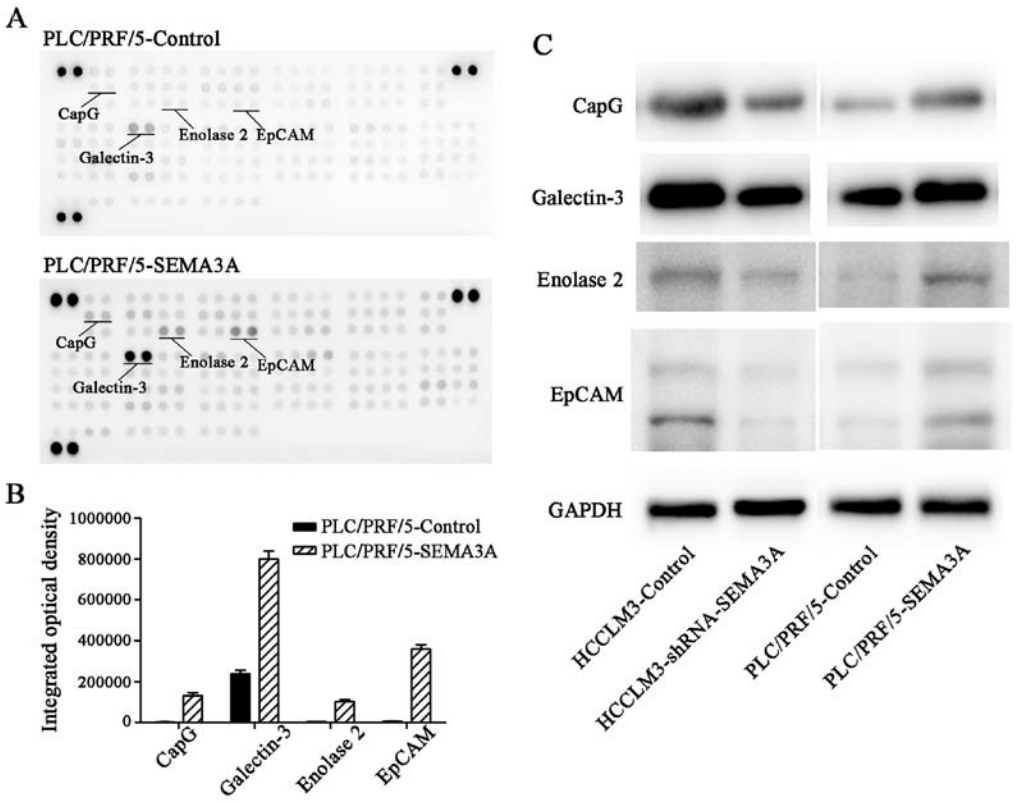

Figure 4. SEMA3A increases CapG, galectin-3, enolase 2 and EpCAM in HCC. (A and B) Proteome profiler antibody array assay revealed that CapG, galectin-3, enolase 2 and EpCAM were significantly overexpressed in the PLC/PRF/5-SEMA3A cells (P<0.05). (C) Western blotting ascertained that the expression levels of CapG, galectin-3, enolase 2 and EpCAM were markedly decreased in the HCCLM3-shRNA-SEMA3A cells, but increased in the PLC/PRF/5-SEMA3A cells. SEMA3A, semaphorin 3A; HCC, hepatocellular carcinoma; CapG, gelsolin-like capping protein; EpCAM, epithelial cell adhesion molecule.

galectin-3, enolase 2 and EpCAM (P<0.05) (Fig. 4A and B). Furthermore, western blot analyses confirmed that SEMA3A upregulated the expression of CapG, galectin-3, enolase 2 and EpCAM in the PLC/PRF/5 cells, and the downregulation of SEMA3A by shRNA in HCCLM3 cells caused a distinct decrease in those cancer-related proteins (Fig. 4C).

SEMA3A promotes the growth and metastasis of HCC in vivo. To validate the observations obtained from the *in vitro* studies, we used a metastatic HCC model in mice to clarify the effects of SEMA3A on tumor growth and metastasis of HCC *in vivo*. After orthotopic transplantation of subcutaneous tumor tissues derived from the HCC cell lines into the livers of nude mice, all of the groups successfully formed liver tumors. We found that the liver localized tumor size in the HCCLM3-shRNA-SEMA3A group was markedly smaller than that in the HCCLM3-control group (1.45±0.28 vs. 3.41±0.51 cm^3, respectively; P<0.01) (Fig. 5A). Similarly, the liver localized tumor size in the PLC/PRF/5-SEMA3A group was 0.75±0.19 cm^3, significantly larger than that in the PLC/PRF/5-control group (0.21±0.03 cm^3; P<0.05) (Fig. 5B). Pulmonary metastasis occurred in 100% (6/6) of the HCCLM3-control nude mice (Fig. 5C), much higher than was observed in the HCCLM3-shRNA-SEMA3A mice (2 out of 6, 33%) (Fig. 5D). Furthermore, the number of pulmonary metastatic nodules of each grade was also greater in the HCCLM3-control mice (P<0.05) (Fig. 5F and G). In addition, only one of the PLC/PRF/5-control mice had pulmonary metastasis (1 out of 6, 17%), a distinctly lower rate than that observed in the PLC/PRF/5-SEMA3A group (4 out of 6, 67%) (Fig. 5E). Moreover, the number of pulmonary metastatic nodules of each grade was also obviously smaller in the PLC/PRF/5-control group (P<0.05) (Fig. 5H). Collectively, these data demonstrate an important role for SEMA3A in HCC progression and metastasis.

Discussion

Hepatocellular carcinoma (HCC) is one of the most lethal cancers, and few effective therapeutic methods are currently available (21). Understanding the molecular mechanisms for HCC progression provides strategies for the intervention and treatment of HCC and improves the survival and prognosis of HCC patients (22). In the present study, we found that the expression of SEMA3A was elevated in HCC tissues compared with the corresponding adjacent non-tumorous liver tissues (ANLTs). These findings agree with the results derived from other solid tumors, including pancreatic, lung, and prostate cancers (12,13,15). Therefore, clarifying the effect and mechanism of SEMA3A on HCC provides the basis for further research and is helpful for developing a novel, effective and practical diagnostic and therapeutic method for HCC.

Recent studies reported that increased expression of SEMA3A is positively related to advanced malignant grade, cell migration and metastatic potential (12,15). However, it remains unclear whether SEMA3A contributes to the progression of HCC. In the present study, we confirmed for the first time that both SEMA3A mRNA and protein are

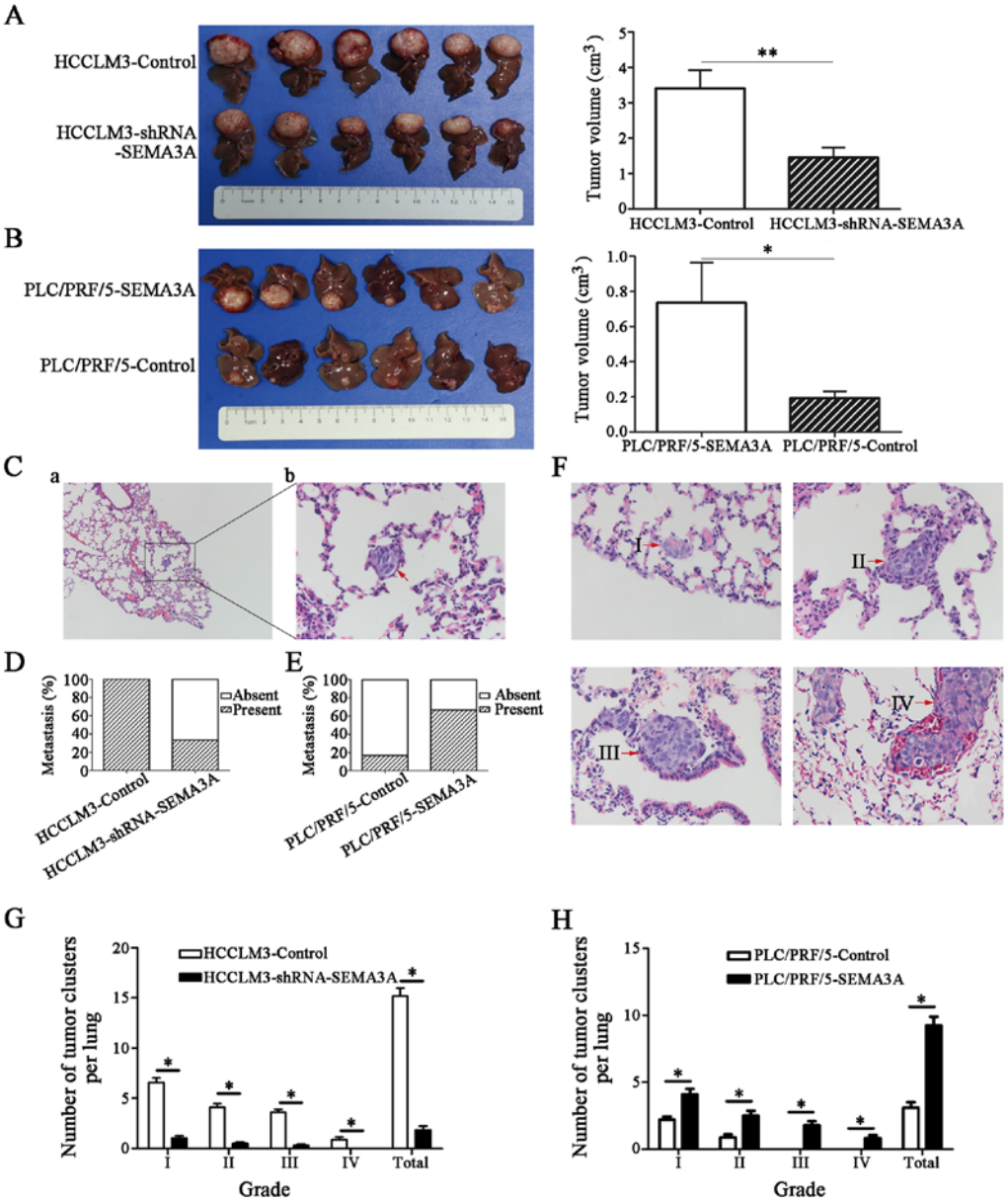

Figure 5. SEMA3A facilitates HCC growth and metastasis *in vivo*. (A) The downregulation of SEMA3A significantly inhibited tumor growth in an orthotopic HCCLM3 cell implantation mouse model (**P<0.01). (B) Overexpression of SEMA3A significantly promoted tumor growth in an orthotopic PLC/PRF/5 cell implantation mouse model (*P<0.05). (C) Representative hematoxylin and eosin (H&E) stained images of metastatic nodules in mouse lungs (a, magnification, x100; b, x400). (D) The pulmonary metastasis rate was lower in the HCCLM3-shRNA-SEMA3A group than in the control group. (E) The pulmonary metastasis rate was distinctly higher in the PLC/PRF/5-SEMA3A group than in the control group. (F) Representative H&E stained images of pulmonary metastatic nodules for grades I-IV (magnification, x400). Classification of lung metastatic nodules was based on the number of tumor cells in the metastatic lesion. (G) Both the number and size of the pulmonary metastases in HCCLM3-shRNA-SEMA3A mice were significantly decreased compared to HCCLM3-control mice (*P<0.05). (H) The number of pulmonary metastatic nodules of each grade was significantly greater in the PLC/PRF/5-SEMA3A group than in the control group (*P<0.05). SEMA3A, semaphorin 3A; HCC, hepatocellular carcinoma.

significantly overexpressed in HCC tissues compared with the corresponding ANLTs, which indicates that SEMA3A may play an important role in HCC progression. Moreover, we demonstrated the expression of SEMA3A in HCC cell lines and found that SEMA3A expression was positively correlated with the invasive and metastatic potential of HCC cells. Our data established for the first time that SEMA3A is upregulated in HCC and may play a critical role in the invasion and metastasis of HCC.

To gain insight on the effect of SEMA3A on the biological behavior of HCC cells, we transfected the corresponding lentivirus into HCC cells to knock down the SEMA3A expression in HCCLM3 cells and overexpress SEMA3A in PLC/PRF/5 cells. We found that upregulation of SEMA3A resulted in a marked promotion of cell proliferation, migration and invasion of PLC/PRF/5 cells *in vitro*, but depletion of SEMA3A led to obvious inhibition of cell proliferation, migration and invasion in HCCLM3 cells. These findings confirmed that SEMA3A promoted the proliferation and invasion of HCC cells.

Current studies ascertained that SEMA3A, as a secreted protein, interacts with neuropilins and plexins, adjusting the expression of cancer-related proteins, to regulate cell shape, adhesion, locomotion and invasion (23-26). Our results indicated that the overexpression of SEMA3A upregulated the

expression of CapG, galectin-3, enolase 2 and EpCAM in PLC/PRF/5 cells. Conversely, SEMA3A depletion attenuated these proteins in HCCLM3 cells. Numerous studies have confirmed that CapG, galectin-3, enolase 2 and EpCAM can induce tumor progression (27-30). These results revealed that SEMA3A promotes the proliferation and invasion of HCC cells by facilitating CapG, galectin-3, enolase 2 and EpCAM expression.

The role of SEMA3A in the modulation of HCC cell proliferation and invasion was further confirmed by *in vivo* assays, in which we demonstrated that the overexpression of SEMA3A was associated with an increased volume of orthotopic tumors. Conversely, knockdown of SEMA3A inhibited orthotopic tumor growth. Moreover, the number and size of pulmonary metastatic lesions in the SEMA3A-overexpressing group were all significantly increased compared with those in the control group, and depletion of SEMA3A suppressed pulmonary metastasis in an HCC metastatic mouse model, suggesting a critical role for SEMA3A in the regulation of the metastasis of HCC.

In conclusion, the present study, reveals for the first time that SEMA3A is overexpressed in HCC. Furthermore, we demonstrated the critical role of SEMA3A in promoting HCC growth, invasion, and metastasis by increasing the expression of CapG, galectin-3, enolase 2 and EpCAM. Collectively, these features of SEMA3A make it a potential therapeutic target for HCC.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81472218, 81272724, 81402376 and 81301821).

References

1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
3. Ou DP, Yang LY, Huang GW, Tao YM, Ding X and Chang ZG: Clinical analysis of the risk factors for recurrence of HCC and its relationship with HBV. World J Gastroenterol 11: 2061-2066, 2005.
4. Kolodkin AL, Matthes DJ, O'Connor TP, Patel NH, Admon A, Bentley D and Goodman CS: Fasciclin IV: Sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron 9: 831-845, 1992.
5. Kolodkin AL, Matthes DJ and Goodman CS: The *semaphorin* genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 75: 1389-1399, 1993.
6. Yazdani U and Terman JR: The semaphorins. Genome Biol 7: 211, 2006.
7. Hung RJ, Yazdani U, Yoon J, Wu H, Yang T, Gupta N, Huang Z, van Berkel WJ and Terman JR: Mical links semaphorins to F-actin disassembly. Nature 463: 823-827, 2010.
8. Ito T, Kagoshima M, Sasaki Y, Li C, Udaka N, Kitsukawa T, Fujisawa H, Taniguchi M, Yagi T, Kitamura H, *et al*: Repulsive axon guidance molecule Sema3A inhibits branching morphogenesis of fetal mouse lung. Mech Dev 97: 35-45, 2000.
9. Maione F, Capano S, Regano D, Zentilin L, Giacca M, Casanovas O, Bussolino F, Serini G and Giraudo E: Semaphorin 3a overcomes cancer hypoxia and metastatic dissemination induced by antiangiogenic treatment in mice. J Clin Invest 122: 1832-1848, 2012.
10. Nasarre P, Gemmill RM and Drabkin HA: The emerging role of class-3 semaphorins and their neuropilin receptors in oncology. Onco Targets Ther 7: 1663-1687, 2014.
11. Bagci T, Wu JK, Pfannl R, Ilag LL and Jay DG: Autocrine semaphorin 3a signaling promotes glioblastoma dispersal. Oncogene 28: 3537-3550, 2009.
12. Müller MW, Giese NA, Swiercz JM, Ceyhan GO, Esposito I, Hinz U, Büchler P, Giese T, Büchler MW, Offermanns S, *et al*: Association of axon guidance factor semaphorin 3a with poor outcome in pancreatic cancer. Int J Cancer 121: 2421-2433, 2007.
13. Zhou H, Wu A, Fu W, Lv Z and Zhang Z: Significance of semaphorin-3A and MMP-14 protein expression in non-small cell lung cancer. Oncol Lett 7: 1395-1400, 2014.
14. Blanc V, Nariculam J, Munson P, Freeman A, Klocker H, Masters J and Williamson M: A role for class 3 semaphorins in prostate cancer. Prostate 71: 649-658, 2011.
15. Herman JG and Meadows GG: Increased class 3 semaphorin expression modulates the invasive and adhesive properties of prostate cancer cells. Int J Oncol 30: 1231-1238, 2007.
16. Rivera LB and Bergers G: Location, location, location: Macrophage positioning within tumors determines pro- or antitumor activity. Cancer Cell 24: 687-689, 2013.
17. Li Y, Tian B, Yang J, Zhao L, Wu X, Ye SL, Liu YK and Tang ZY: Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive in vivo selection and studies on metastatic characteristics. J Cancer Res Clin Oncol 130: 460-468, 2004.
18. Ye QH, Qin LX, Forgues M, He P, Kim JW, Peng AC, Simon R, Li Y, Robles AI, Chen Y, *et al*: Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 9: 416-423, 2003.
19. Sun FX, Tang ZY, Lui KD, Ye SL, Xue Q, Gao DM and Ma ZC: Establishment of a metastatic model of human hepatocellular carcinoma in nude mice via orthotopic implantation of histologically intact tissues. Int J Cancer 66: 239-243, 1996.
20. Dai Z, Zhou SL, Zhou ZJ, Bai DS, Xu XY, Fu XT, Chen Q, Zhao YM, Zhu K, Yu L, *et al*: Capn4 contributes to tumour growth and metastasis of hepatocellular carcinoma by activation of the FAK-Src signalling pathways. J Pathol 234: 316-328, 2014.
21. Oliveri RS, Wetterslev J and Gluud C: Hepatocellular carcinoma. Lancet 380: 470-471, 2012.
22. Villanueva A, Newell P, Chiang DY, Friedman SL and Llovet JM: Genomics and signaling pathways in hepatocellular carcinoma. Semin Liver Dis 27: 55-76, 2007.
23. Suzuki K, Kumanogoh A and Kikutani H: Semaphorins and their receptors in immune cell interactions. Nat Immunol 9: 17-23, 2008.
24. Wang Y, He H, Srivastava N, Vikarunnessa S, Chen YB, Jiang J, Cowan CW and Zhang X: Plexins are GTPase-activating proteins for Rap and are activated by induced dimerization. Sci Signal 5: ra6, 2012.
25. Lepelletier Y, Smaniotto S, Hadj-Slimane R, Villa-Verde DM, Nogueira AC, Dardenne M, Hermine O and Savino W: Control of human thymocyte migration by Neuropilin-1/Semaphorin-3A-mediated interactions. Proc Natl Acad Sci USA 104: 5545-5550, 2007.
26. Garcia F, Lepelletier Y, Smaniotto S, Hadj-Slimane R, Dardenne M, Hermine O and Savino W: Inhibitory effect of semaphorin-3A, a known axon guidance molecule, in the human thymocyte migration induced by CXCL12. J Leukoc Biol 91: 7-13, 2012.
27. Westbrook JA, Cairns DA, Peng J, Speirs V, Hanby AM, Holen I, Wood SL, Ottewell PD, Marshall H, Banks RE, *et al*: CAPG and GIPC1: Breast cancer biomarkers for bone metastasis development and treatment. J Natl Cancer Inst 108: pii: djv360, 2016.
28. Wang L and Guo XL: Molecular regulation of galectin-3 expression and therapeutic implication in cancer progression. Biomed Pharmacother 78: 165-171, 2016.
29. Tang D, Wang M, Sui A, Wang Y, Yang R, Wang Z, Zhao Y, Jiao W and Shen Y: Prospective validation of quantitative NSE mRNA in pleural fluid of non-small cell lung cancer patients. Med Oncol 30: 699, 2013.
30. Gires O and Bauerle PA: EpCAM as a target in cancer therapy. JJ Clin Oncol 28: e239-e242, 2010.