Sema3C promotes hepatic metastasis and predicts poor prognosis in gastric adenocarcinoma

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Abstract
Objective: Semaphorin 3C (Sema3C) may regulate tumor metastasis and prognosis. We determined the biological roles of Sema3C in the hepatic metastasis of gastric adenocarcinoma and evaluated its clinical significance as a potential biomarker.
Methods: Sema3C expression in gastric cancer (GC) cell lines and tissues was measured using RT-qPCR and western blotting. Moreover, Sema3C functions were analyzed using Transwell assays and in vitro metastasis assays in gain- and loss-of-function experiments. Furthermore, the impact of Sema3C on the prognosis of 80 randomly selected patients with GC was investigated by immunohistochemistry. Additionally, the expression of epithelial-mesenchymal transition (EMT) indicators was verified by immunohistochemistry in GC tissues.
Results: Sema3C expression was significantly upregulated in highly metastatic GC cell lines and tissues. Additionally, Sema3C promoted invasion, migration and hepatic metastasis in GC cells. Moreover, Sema3C expression was positively correlated with clinicopathological features in GC and paired hepatic metastatic tissues, and Sema3C expression was an independent prognostic factor. Finally, Sema3C expression was associated with node metastasis, hepatic metastasis and EMT marker expression.
Conclusions: Sema3C may play roles in regulating the EMT and metastasis of gastric adenocarcinoma, highlighting its potential use as a prognostic factor for hepatic metastasis and poor prognosis in gastric adenocarcinoma.

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Introduction

Gastric cancer (GC) is one of the most threatening malignant tumors. According to 2018 statistics, the number of new gastric cancer cases exceeded 1,000,000 worldwide, and the population in East Asia accounted for approximately 47%. Globally, the number of patients who died of GC was 783,000, making it the third deadliest malignancy after lung cancer and colorectal cancer.\textsuperscript{1,2} Early diagnosis and treatment can significantly increase the survival rate of patients with GC. However, in China, most patients present with advanced stages at the time of diagnosis, and the average survival period of these patients is less than 1 year. Hepatic metastasis is one of the most common hematogenous metastases of GC and represents one of the primary causes of death in patients with GC.\textsuperscript{3}

In recent years, with the continuous development of new alternative strategies, such as chemotherapy, targeted therapy, surgery and radiofrequency therapy, the quality of life for patients with GC has been partially improved. However, the overall therapeutic effect remains unsatisfactory. Hence, research on the hepatic metastasis of GC has gained increasing interest. Many scholars have investigated the various markers of hepatic metastasis in GC.\textsuperscript{4,5} Unfortunately, the mechanism of hepatic metastasis is quite complex and remains poorly understood.\textsuperscript{6}

Numerous studies have demonstrated that semaphorins are overexpressed in a variety of malignant tumors, including glioma, breast cancer and lung cancer.\textsuperscript{7} According to previous studies, semaphorin 3C (Sema3C) could promote the formation and metastasis of neuroblastoma by inducing a pro-metastatic microenvironment.\textsuperscript{8} Sema3C is a member of the semaphorin family that serves important roles in diverse physiological processes, including axonal growth, immune responses, cell adhesion, migration and bone remodeling.\textsuperscript{9} The expression level of Sema3C is related to the occurrence and development of several tumors.\textsuperscript{10,11} However, the relationship between Sema3C and GC is not well understood. Therefore, we aimed to determine the biological roles of Sema3C in the hepatic metastasis of GC and evaluate its clinical significance as a potential disease biomarker.

Materials and Methods

Patients

Eighty freshly collected GC specimens paired with adjacent normal gastric tissues and hepatic metastatic tissues were obtained from gastrectomy surgeries performed from January 2009 to August 2014. This retrospective study was approved by the Research Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, China (ethical approval number: 20171114) and performed in accordance with the ethical standards of the World Medical Association’s Declaration of Helsinki.
Signed informed consent forms were obtained from all the patients included in this study. All tissues were immediately frozen in liquid nitrogen and maintained at −80°C until RNA and DNA extraction for RT-qPCR were performed. For Sema3C immunohistochemical evaluation, the inclusion criteria for patients with GC were as follows: 1) a pathologic diagnosis of gastric adenocarcinoma; 2) no radiotherapy, chemotherapy or other anti-cancer therapies prior to the surgery; and 3) availability of complete clinicopathological and clinical follow-up data. The final date of follow-up was 31 December 2016 for all cases examined. Eighty paraffin-embedded tissue samples met the inclusion criteria and were collected from patients at the Department of Gastrointestinal Surgery, RenJi Hospital, Shanghai Jiao tong University, School of Medicine from January 2009 to August 2014 for tissue microarray (TMA) construction and immunohistochemistry staining. Overall survival (OS) was calculated from the date of tumor resection until death or the date of the last follow-up.

**Cell culture and reagents**

The human GC cell lines AGS, BGC-823, HGC–27, MGC–803, MKN-28, MKN–45, N87, SGC–7901 and GES-1 were all maintained in the Shanghai Cancer Institute, Ren Ji Hospital. According to ATCC protocols, all cells were cultured in RPMI-1640 medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) or F–12 Medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) and maintained at 37°C in a humidified incubator with 5% CO₂. The cell medium was replaced every 2 to 3 days, and 1X phosphate buffer solution (PBS) was used for cell washing prior to the medium being replaced. Cells were collected in the logarithmic growth phase for protein and RNA extraction.

**Total RNA extraction and RT-qPCR**

Total RNA was extracted from 80 fresh cancer tissue samples and paired normal tissues using Trizol reagent (Takara, Dalian, China) following the manufacturer’s instructions. The reverse-transcription reactions were carried out with random primers and M-MLV Reverse Transcriptase (Takara, Dalian, China), and the resulting cDNA used for RT-qPCR reactions via the SYBR-Green method. All qPCR reactions were performed on a StepOneTM RT-qPCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: GAPDH forward, 5′-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5′-GAA GAT GGT GAT GGG ATT TC-3' and Sema3C forward, 5′-GCGAAGCA-GCATGAGGTGTATTGGA-3′ and reverse, 5′-CGATGTAGTTGTGGCACTCTGTC TG-3′. The 2-ΔCt method was used to quantify the relative Sema3C expression levels.

**TMA construction**

TMAs were constructed by Shanghai Zhuo Li Biotechnology Co., Ltd (Zhuo Li Biotechnology Co, Shanghai, China). Because GC tissue is dispersed within the interstitial space, GC sample staining is necessary. Consequently, tissue paraffin blocks of GC samples were stained with hematoxylin and eosin to confirm the diagnoses, and the most typical histological characteristics were marked under a microscope. Two 1.5-mm cores per donor block were transferred into a recipient block TMA, and each dot array contained fewer than 160 dots. Three-micron-thick sections were cut from
the recipient block and transferred onto glass slides using an adhesive tape transfer system for ultraviolet cross-linkage.

**Immunohistochemistry**

The TMA glass slides were baked at 55°C for 1 hour, then de-paraffinized gradually in xylene, 50% xylene and gradient concentrations of ethanol, followed by immersion in tap water. Peroxidase activity was blocked with 0.3% hydrogen peroxide at 37°C for 30 minutes. Antigen retrieval was carried out by boiling sections in 10 mmol/L citrate buffer (pH 6.0) for 15 minutes. Then, the tissues were incubated with a Sema3C antibody (diluted 1:100, mouse monoclonal anti-Sema3C antibody [3D1] ab128062, Abcam, Cambridge, MA, USA) overnight at 4°C. The next day, the tissues were washed with PBS three times and incubated with a goat anti-rabbit IgG-horseradish peroxidase (HRP) (HUABIO, Woburn, MA, USA) or goat anti-mouse IgG-HRP (Abcam) secondary antibody for 1 hour at room temperature. Immunostaining was performed using the diaminobenzidine chromogen substrate method (Dako, Carpinteria, CA, USA), and the chromogenic reaction was controlled under a microscope. After immunostaining, tissues were immersed in hematoxylin for nuclear staining. The slides were then dehydrated through gradient concentrations of ethanol, cleared with xylene and coverslipped with neutral balsam. TMAs were scored as follows: absence of any staining = 0, weak-to-strong complete nuclear staining in <25% of tumor cells = 1, weak-to-strong complete nuclear staining in 25% to 50% of tumor cells = 2 and strong complete nuclear staining in ≥50% of tumor cells = 3. Scores between 0 and 1 were considered low expression, and a score of 2 to 3 was considered high expression for subsequent statistical analyses. All scoring was performed by two independent pathologists who were blinded to the clinical outcomes.

**Western blot analysis**

Total proteins were prepared from fresh frozen tissue or cultured cell samples by complete cell lysis (Roche Mannheim, Germany) with protease and phosphatase inhibitors. Cytoplasmic proteins and nuclear proteins were isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China). Denatured proteins (20–50 μg) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. The following primary antibodies were used: vinculin (Millipore, Darmstadt, Germany), β-actin (Sigma-Aldrich Inc., St. Louis, MO, USA), β-catenin (BD Bioscience, San Jose, CA, USA), histone (Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin (E-cad) (Cell Signaling Technology, Boston, MA, USA) and vimentin (VIM) (Santa Cruz). The bands were scanned using the ChemiDocXRS+ Imaging System (Bio-Rad, Hercules, CA, USA) and quantified using Quantity One v4.6.2 software (Bio-Rad).

**SiRNA transfections**

GC cells were 50% to 70% confluent when transfected. Sema3C was silenced using specific siRNAs with the following sequences: si-1, GCGCUACUAAUUGGAAGATT and si-2, GGGCUGAGGACCUCUGCAG AAGATT. Scrambled siRNA targeting no known gene sequence was used as the negative control. All RNAs were obtained from Genechem (China). Lipofectamine® RNAiMAX reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to conduct siRNA transfections in accordance with the manufacturer’s
protocol, and the transfection efficiency was detected by qPCR and western blot.

**CCK-8 assay**

The effect of Sema3C knockdown on GC cell proliferation was accessed using a CCK-8 assay (Dojindo, Kumamoto, Japan). At 24 hours prior to siRNA transfection, GC cells in the logarithmic growth phase were seeded at 2000 cells/well in 96-well plates containing 100μl medium. At the indicated time points (24, 48, 72, 94 and 120 hours following transfection), 10μl CCK-8 were added into each well, and the plates were incubated at 37°C for another 3 hours. The absorbance (optical density; OD) was measured at a wavelength of 450 nm. The experiment was performed in triplicate. The relative growth rate was calculated as follows: Relative growth rate (\%) = (OD of treated cells/OD of control cells) × 100.

**Colony formation assay**

Five hundred cells were seeded in six-well plates. After incubation for 2 weeks, colonies were fixed with 4% paraformaldehyde (Servicebio, Wuhan, China) and stained with 0.2% crystal violet (Servicebio), and then the number of clones formed was counted.

**Invasion and migration assay**

The effect of Sema3C knockdown on GC cell migration was determined using a Transwell migration assay. GC cells were plated at 2.5 × 10^5/well in six-well plates in the presence or absence of Matrigel. Following transfection with siRNA for 72 hours, cells (5 × 10^4) in 800μl serum-free medium were seeded in the upper chamber of 12-well plates, and 1mL DMEM containing 10% FBS was placed in the lower chamber. Following incubation for 24 hours, the upper chamber was washed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes. Cells on the top of the membrane were removed with cotton swabs. Cells that migrated to the bottom were stained with 0.1% crystal violet for 15 minutes. Four randomly selected fields of the membrane were counted under an inverted microscope (magnification, ×100).

**Statistical analysis**

Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (San Diego, CA, USA) software. Sema3C mRNA levels in gastric carcinomas, paired noncancerous and hepatic metastatic tissues were compared using a paired sample t-test. The chi-square test was used to analyze the relationship between Sema3C expression and clinicopathological characteristics. OS curves were calculated according to Kaplan–Meier method, and the log-rank test was used for comparing the survival distributions. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. All statistical tests were two-sided. P values less than 0.05 were considered statistically significant.

**Results**

**Sema3C expression in 80 gastric carcinoma, noncancerous and hepatic metastatic tissues**

Three gastric carcinoma tissues, matched noncancerous tissues and hepatic metastatic tissues were obtained, and gene expression profile sequencing was performed. Sema3C expression was significantly increased in hepatic metastatic tissues and gastric carcinoma tissues compared with matched noncancerous tissues. Sema3C was the most
upregulated gene among the axonal guidance factors (P < 0.01) (Figures 1a–1b).

Sema3C mRNA expression levels in gastric carcinoma tissues, matched noncancerous tissues and hepatic metastatic tissues from 80 patients were examined by qRT-PCR. The histogram of relative mRNA expression levels showed generally low transcript levels of Sema3C in the noncancerous tissues compared with gastric tissues and hepatic metastatic tissues (Figure 1c).

**Correlation of Sema3C expression with clinicopathological features in 80 gastric carcinomas**

Next, we performed immunohistochemistry staining in 80 GC samples. Representative images of Sema3C immunostaining scored as 0, 1, 2 and 3 were shown in Figure 2a. The correlation of Sema3C expression with clinicopathological features was analyzed and shown in Table 1. Tumor size

![Figure 1](image-url)
(P = 0.023), nodal stage (P = 0.008), vessel invasion (P = 0.036) and nerve invasion (P = 0.003) were found to be significantly correlated with Sema3C expression. However, no significant difference was found regarding age, sex, tumor location, Lauren classification and tumor depth. Univariate Cox proportional hazards regression analysis indicated that Sema3C expression (P < 0.0001), tumor size

Figure 2. Association between Sema3C expression and prognosis in gastric cancer (GC). (a) Immunohistochemistry staining was performed in 80 GC samples, and representative images of Sema3C immunostaining were shown. The degree of staining was scored as follows: absence of any staining = 0 (Negative), weak-to-strong complete nuclear staining in < 25% of tumor cells = 1 (Weak), weak-to-strong complete nuclear staining in 25% to 50% of tumor cells = 2 (Moderate) and strong complete nuclear staining in ≥ 50% of tumor cells = 3 (Positive). Scores between 0 and 1 were considered low expression, and a score of 2 to 3 was considered high expression. (b) Survival analysis of samples from the TCGA database showed that the patients with higher Sema3C expression had poorer overall survival. (c) Kaplan–Meier and long-rank analysis of 80 GC samples revealed that patients with higher Sema3C expression had a poorer overall survival
(P < 0.0001), nerve invasion (P < 0.0001) and vascular invasion (P = 0.036) were hazardous prognostic factors for the OS of patients with GC. Meanwhile, multivariate Cox proportional hazards regression analysis identified Sema3C expression (P = 0.014) and nerve invasion (P < 0.0001) as independent predictors of OS (Table 2). The OS of 415 patients with GC in the TCGA database was shown in Figure 2b, which demonstrated that high Sema3C expression was correlated with poor prognosis (P = 0.019). Similarly, Sema3C expression was significantly associated with prognosis in our GC cohort (P = 0.0374) (Figure 2c).

### Table 1. Correlations between Sema3C expression and clinicopathological parameters in 80 patients with gastric cancer.

|                          | Number | Sema3C low expression | Sema3C high expression | P value |
|--------------------------|--------|-----------------------|------------------------|---------|
| **Sex**                  |        |                       |                        |         |
| Male                     | 63     | 21                    | 42                     | 0.091   |
| Female                   | 17     | 10                    | 7                      |         |
| **Age**                  |        |                       |                        |         |
| ≤60                      | 26     | 8                     | 18                     | 0.339   |
| >60                      | 54     | 23                    | 31                     |         |
| **Tumor location**       |        |                       |                        |         |
| Cardia                   | 25     | 10                    | 15                     | 0.995   |
| Non-cardia               | 55     | 21                    | 34                     |         |
| **Tumor size**           |        |                       |                        |         |
| ≤5 cm                    | 25     | 14                    | 11                     | 0.023   |
| >5 cm                    | 55     | 15                    | 40                     |         |
| **Lauren classification**|        |                       |                        | 0.606   |
| Intestinal               | 47     | 20                    | 27                     |         |
| Diffuse                  | 25     | 6                     | 19                     |         |
| Mix                      | 8      | 2                     | 6                      |         |
| **Depth of tumor (pT)**  |        |                       |                        | 0.147   |
| T1                       | 1      | 0                     | 1                      |         |
| T2                       | 2      | 2                     | 0                      |         |
| T3                       | 0      | 0                     | 0                      |         |
| T4                       | 77     | 29                    | 48                     |         |
| **Nodal stage (pN)**     |        |                       |                        | 0.008   |
| N0                       | 9      | 7                     | 2                      |         |
| N1                       | 8      | 5                     | 3                      |         |
| N2                       | 21     | 9                     | 12                     |         |
| N3                       | 42     | 10                    | 32                     |         |
| **Vessel invasion**      |        |                       |                        | 0.036   |
| Negative                 | 47     | 23                    | 24                     |         |
| Positive                 | 33     | 8                     | 25                     |         |
| **Nerve invasion**       |        |                       |                        | 0.003   |
| Negative                 | 60     | 29                    | 31                     |         |
| Positive                 | 20     | 2                     | 18                     |         |

Statistical significance: P < 0.05.
Sema3C knockdown inhibits GC cell proliferation, invasion and migration

Based on qPCR analysis, AGS and N87 cell lines exhibited the highest expression of Sema3C and were thus selected for further analysis (Figure 3a).

To understand the function of Sema3C in GC, the effect of Sema3C knockdown on GC cell proliferation was examined. The results of CCK-8 and colony formation assays demonstrated that the relative growth rate was significantly decreased in Sema3C siRNA-transfected cells compared with negative control (siRNA-NC) cells (all P < 0.05) (Figures 3b–3e). These results suggested that Sema3C knockdown could inhibit GC cell proliferation.

Transwell invasion and migration assays demonstrated that the number of migrated cells was significantly decreased following Sema3C knockdown compared with the number of migrated cells in the siRNA-NC groups (all P < 0.001) (Figures 3f–3i).

Sema3C promotes EMT in gastric carcinoma

To investigate the hypothesis that Sema3C regulates EMT, we detected the expression of the epithelial differentiation marker E-cad and the mesenchymal marker VIM in GC cells and tissues. Silencing Sema3C in N87 and AGS cells increased the subcellular expression of E-cad and downregulated the expression of VIM (Figure 4a). To evaluate whether these results could be applied to the clinical setting, immunohistochemistry analysis of tissue arrays was conducted. The results revealed strong expression of VIM and weak expression of E-cad in GC tissues with high Sema3C expression (Figure 4b), indicating that EMT may affect the formation of GC and that Sema3C may regulate EMT in GC.

| Factors                                      | Univariate          | Multivariate         |
|----------------------------------------------|---------------------|----------------------|
|                                               | HR (95% CI)         | P value              | HR (95% CI)         | P value              |
| Age                                           | 0.935 (0.544–1.605) | 0.806                | 0.935 (0.544–1.605) | 0.806                |
| Sex                                           | 0.712 (0.383–1.323) | 0.282                | 0.712 (0.383–1.323) | 0.282                |
| Location (cardia vs. non-cardia)              | 1.341 (0.782–2.302) | 0.287                | 1.341 (0.782–2.302) | 0.287                |
| Lauren’s classification (intestinal vs. non-intestinal) | 1.110 (0.656–1.879) | 0.697                | 1.110 (0.656–1.879) | 0.697                |
| Size (>5 cm vs ≤5 cm)                         | 0.320 (0.173–0.593) | &lt;0.0001           | 0.519 (0.263–1.027) | 0.060                |
| T stage (T1–2 vs. T3–4)                      | 0.333 (0.046–2.405) | 0.276                | 0.333 (0.046–2.405) | 0.276                |
| N stage (N0 vs. N1–3)                        | 0.695 (0.314–1.538) | 0.370                | 0.695 (0.314–1.538) | 0.370                |
| Nerve invasion (invasion vs. non-invasion)    | 0.170 (0.087–0.333) | &lt;0.0001           | 0.266 (0.135–0.523) | &lt;0.0001           |
| Vascular invasion (invasion vs. non-invasion) | 0.571 (0.338–0.965) | 0.036                | 0.778 (0.446–1.359) | 0.378                |
| Sema3C expression (high vs. low)              | 4.459 (2.420–8.217) | &lt;0.0001           | 2.472 (1.203–5.078) | 0.014                |

Table 2. Univariate and multivariate Cox regression analysis of potential prognostic factors in gastric cancer.

Statistical significance: P < 0.05.
HR, hazard ratio; CI, confidence interval.
Hepatic metastasis is one of the most distinguished phenotypes of GC, and it results in extremely poor prognosis and relatively high recurrence rates. Semaphorins are a family of secreted proteins that have been identified as novel tumor-associated factors. Based on their structural similarity, semaphorins are divided into eight classes of 25 proteins. Semaphorins have a Sema domain of approximately 500 amino acids located near the N terminus of their proteins.

Figure 3. Sema3C promoted gastric cancer cell proliferation, invasion and migration. (a) Relative mRNA levels of Sema3C in a panel of gastric cell lines (N87, AGS, HGC27, MKN45, MKN28, BGC-823, MGC-803, SGC-7901 and GES-1). (b–e) CCK8 assays and colony formation assays in AGS and N87 cells following Sema3C silencing showed that Sema3C knockdown significantly inhibited the growth of gastric cancer cells. *P < 0.05, **P < 0.01, ***P < 0.001. (f–i) Transwell assays showed that Sema3C-depleted AGS and N87 cells exhibited a reduced migratory (without Matrigel) and invasive (with Matrigel) capacity. ***P < 0.001 (Sema3C NC-Sema3C negative control; Sema3C si1-Sema3C silenced group 1; Sema3C si2-Sema3C silenced group 2). All results are shown as the mean ± standard deviation, and all data were statistically analyzed by paired t-tests.

**Discussion**

Hepatic metastasis is one of the most distinguished phenotypes of GC, and it results in extremely poor prognosis and relatively high recurrence rates. Semaphorins are a family of secreted proteins that have been
ectodomain, and the structure of semaphorins extends approximately 70 amino acids. These characteristics provide Semaphorins with their unique biological activity. Semaphorins generally act on the actin cytoskeleton and adhesion plaques. Semaphorin signaling affects adhesion plaque assembly and induces cytoskeletal remodeling, thereby affecting cell shape, extracellular matrix adhesion, cell mobility and cell migration.\textsuperscript{14}

Previous studies have demonstrated that Sema3C promotes endotheliocyte migration, cancer metastasis and angiogenesis in the tumor microenvironment.\textsuperscript{15–17} However, the functions of Sema3C in regulating GC cell growth and migration remain unknown.

Nine cell lines, including N87, AGS, BGC823, MKN–45 and SGC–7901, were selected for screening. These are commonly used in GC studies.\textsuperscript{18,19} Finally, N87 and AGS cell lines were selected based on the expression level of Sema3C. In this study, we demonstrated that the expression of Sema3C in N87 and AGS GC cell lines was suppressed using RNAi. The CCK8 test confirmed that Sema3C expression was closely related to the proliferation of GC cells, indicating that the abnormally high levels of Sema3C could significantly increase the proliferative ability of GC cells.

**Figure 4.** Sema3C promotes epithelial-to-mesenchymal transition in gastric carcinoma. (a) Western blot analysis showed that silencing Sema3C in N87 and AGS cells increased the subcellular expression of E-cadherin and downregulated the expression of Vimentin. B-actin was used as the control. (b) Immunohistochemistry of tissue arrays revealed strong expression of Vimentin and weak E-cadherin expression in GC tissues with high Sema3C expression.
cells. Transwell assay results confirmed that the invasion and migration abilities of Sema3C-silenced GC cells were inhibited.

Furthermore, western blotting and immunohistochemistry analyses demonstrated that Sema3C likely promoted GC formation and hepatic metastasis by mediating EMT. EMT, which was first reported in early embryonic morphogenesis,\textsuperscript{20} has been shown to play key roles in cancer progression and metastasis.\textsuperscript{21} During EMT, non-motile, polarized epithelial cells tightly connected by cell–cell junctions dissolve their cell–cell junctions and convert into individual, non-polarized, motile and invasive mesenchymal cells.\textsuperscript{22--24} By immunohistochemistry analysis of GC microarrays, we further identified negative E-cad staining and positive VIM staining in cancer tissues. According to previous literature, aberrant activation of EMT induces the downregulation of E-cad and upregulation of the mesenchymal marker VIM or N-cadherin, which promotes the formation of adhesions between cells and the stroma. These results, together with previous studies, suggest that Sema3C serves an important role in GC growth and hepatic metastasis.

There are some limitations to this study. The data were obtained from a retrospective study, and there might have been bias caused by randomly selected patients and the limited number of patients. Additionally, the mechanisms underlying the interactions among Sema3C, EMT and the hepatic metastasis of gastric adenocarcinoma remain unknown and require more detailed experiments.

In conclusion, we demonstrated that Sema3C is significantly upregulated in cancerous hepatic metastatic cells and tissues. Sema3C overexpression may inhibit GC cell migration, invasion and hepatic metastasis. Furthermore, Sema3C expression is independently associated with poor prognosis in GC, and this might be mediated through enhanced EMT. Therefore, our findings provide evidence that Sema3C is a key regulator of hepatic metastasis in GC and a potential new target in GC therapies.

**Availability of data and materials**

The datasets generated and analyzed during the study are available in The Cancer Genome Atlas (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). Other datasets used in the study are available from the corresponding author upon reasonable request.

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**Declaration of conflicting interest**

The authors declare that there is no conflict of interest.

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**Author contributions**

HC and GZ conceived and designed the study. ML, DX, XX, and BN performed the experiments. ML, and DX wrote the study. CZ, GZ, and HC reviewed and edited the manuscript. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

**References**

1. Information Committee of Korean Gastric Cancer A. Korean gastric cancer association nationwide survey on gastric cancer in 2014. *J Gastric Cancer* 2016; 16: 131–140.

2. Bray F, Ferlay J, Soerjomataram I, et al. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2018; 68: 394–424.
3. Cervantes A, Roda D, Tarazona N, et al. Current questions for the treatment of advanced gastric cancer. Cancer Treat Rev 2013; 39: 60–67.

4. Nasri B, Inokuchi M, Ishikawa T, et al. High expression of EphA3 (erythropoietin-producing hepatocellular A3) in gastric cancer is associated with metastasis and poor survival. BMC Clin Pathol 2017; 17: 8.

5. Xiong J, Li Z, Zhang Y, et al. PRL-3 promotes the peritoneal metastasis of gastric cancer through the PI3K/Akt signaling pathway by regulating PTEN. Oncol Rep 2016; 36: 1819–1828.

6. Jin X, Zhu Z and Shi Y. Metastasis mechanism and gene/protein expression in gastric cancer with distant organs metastasis. Bull Cancer 2014; 101: E1–E12.

7. Miyato H, Tsuno NH and Kitayama J. Semaphorin 3C is involved in the progression of gastric cancer. Cancer Sci 2012; 103: 1961–1966.

8. Delloye-Bourgeois C, Bertin L, Thoinet K, et al. Microenvironment-Driven Shift of Cohesion/Detachment Balance within Tumors Induces a Switch toward Metastasis in Neuroblastoma. Cancer Cell 2017; 32: 427–443.

9. Rizzolio S and Tamagnone L. Semaphorin signals on the road to cancer invasion and metastasis. Cell Adh Migr 2007; 1: 62–68.

10. Xu X, Zhao Z, Guo S, et al. Increased semaphorin 3c expression promotes tumor growth and metastasis in pancreatic ductal adenocarcinoma by activating the ERK1/2 signaling pathway. Cancer Lett 2017; 397: 12–22.

11. Man J, Shoemake J, Zhou W, et al. Sema3C Promotes the Survival and Tumorigenicity of Glioma Stem Cells Through Ral1 Activation. Cell Rep 2014; 9: 1812–1826.

12. Liu R, Shuai Y, Luo J, et al. SEMA3C Promotes Cervical Cancer Growth and Is Associated With Poor Prognosis. Front Oncol 2019; 9: 1035.

13. Neufeld G, Mumblat Y, Smolkin T, et al. The semaphorins and their receptors as modulators of tumor progression. Drug Resist Updat 2016; 29: 1–12.

14. Alto LT and Terman JR. Semaphorins and their Signaling Mechanisms. Methods Mol Biol 2017; 1493: 1–25.

15. Herman JG and Meadows GG. Increased class 3 semaphorin expression modulates the invasive and adhesive properties of prostate cancer cells. Int J Oncol 2007; 30: 1231–1238.

16. Liao YL, Sun YM, Chau GY, et al. Identification of SOX4 target genes using phylogenetic footprinting-based prediction from expression microarrays suggests that overexpression of SOX4 potentiates metastasis in hepatocellular carcinoma. Oncogene 2008; 27: 5578–5589.

17. Esselens C, Malapeira J, Colomé N, et al. The cleavage of semaphorin 3C induced by ADAMTS1 promotes cell migration. J Biol Chem 2010; 285: 2463–2473.

18. Wang L, Yin J, Wang X, et al. C-Type Lectin-Like Receptor 2 Suppresses AKT Signaling and Invasive Activities of Gastric Cancer Cells by Blocking Expression of Phosphoinositide 3-Kinase Subunits. Gastroenterology 2016; 150: 1183–1195.e16.

19. Wu YJ, Lin SH, Din ZH, et al. Sinulariolide Inhibits Gastric Cancer Cell Migration and Invasion through Downregulation of the EMT Process and Suppression of FAK/PI3K/AKT/mTOR and MAPKs Signaling Pathways. Mar Drugs 2019; 17: 668–679.

20. Shook D and Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev 2003; 120: 1351–1383.

21. Poljak K and Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 2009; 9: 265–273.

22. Yilmaz M and Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 2009; 28: 15–33.

23. Tao Y, Yang S, Wu Y, et al. MicroRNA-216a inhibits the metastasis of gastric cancer cells by targeting JAK2/STAT3-mediated EMT process. Oncotarget 2017; 8: 88870–88881.

24. Bu X, Zhang A, Chen Z, et al. Migration of gastric cancer is suppressed by recombinant Newcastle disease virus (rL-RVG) via regulating α7-nicotinic acetylcholine receptors/ERK-EMT. BMC Cancer 2019; 19: 976–988.