Expression of semaphorin 5A and its receptor plexin B3 contributes to invasion and metastasis of gastric carcinoma

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Abstract

AIM: To investigate the protein and mRNA expression of semaphorin 5A and its receptor plexin B3 in gastric carcinoma and explore its role in the invasion and metastasis of gastric carcinoma.

METHODS: Expression of semaphorin 5A and its receptor plexin B3 in 48 samples of primary gastric carcinoma, its corresponding non-neoplastic mucosa, and matched regional lymph node metastasis was assayed by reverse transcription-polymerase chain reaction (RT-PCR), real-time RT-PCR and Western blotting.

RESULTS: The protein and mRNA expression of semaphorin 5A and its receptor plexin B3 increased gradually in non-neoplastic mucosa, primary gastric carcinoma and lymph node metastasis \( (P < 0.05) \). Moreover, the expression of semaphorin 5A was closely correlated with that of plexin B3.

CONCLUSION: Semaphorin 5A and its receptor plexin B3 play an important role in the invasion and metastasis of gastric carcinoma.
are identified as the best characterized semaphorin receptors, which are segregated into four sub-families containing nine members. It has been shown that some vertebrate semaphorins belonging to classes 4-7 can bind directly to plexins and activate plexin-mediated signal transduction\textsuperscript{[2,3]}. These semaphorins and plexins have been originally characterized as constituents of the complex regulatory system responsible for the guidance of axons during the development of the central nervous system\textsuperscript{[4,5]}. However, a growing body of evidence suggests that certain semaphorins, through interacting with its receptors, play a regulatory role in the occurrence and development of tumor\textsuperscript{[6,9]}. Semaphorin 5A is a member of class 5 semaphorins. Plexin B3, belonging to class B plexin subfamily, is a receptor for semaphorin 5A\textsuperscript{[10]}. However, it is unclear whether semaphorin 5A exerts certain biological functions in the progression of human cancers including gastric carcinoma through plexin B3.

In the present study, we investigated the protein and mRNA expression of semaphorin 5A, plexin B3 in primary gastric carcinoma as well as in its corresponding non-neoplastic mucosa and matched regional lymph node metastasis, and preliminarily analyzed their relation with the invasion and metastasis of gastric cancer.

**MATERIALS AND METHODS**

**Patients and specimens**

Forty-eight advanced gastric adenocarcinoma (TNM stage III-IV) patients (28 male and 20 female) with lymph node metastasis diagnosed by postoperative pathology were investigated in this study. Their mean age was 58.7 years (range 45-68 years). The patients received neither chemotherapy nor radiation therapy prior to tumor resection and provided their consent for use of tumor tissue. Tissue blocks of non-neoplastic mucosa (> 5 cm away from the edge of tumor), primary tumor and its corresponding metastatic lymph nodes were obtained within 30 min after they were removed from the patients. Each block was cut into two pieces, one for routine pathologic diagnosis and the other for molecular analysis. Samples were frozen in liquid nitrogen immediately and stored at -260°C until use.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Tissues were lysed using Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated using chloroform and isopropyl alcohol according to the manufacturer's instructions. After RNA was quantified, 1-5 μg of RNA was annealed to Oligo (dT) at 65°C for 5 min and cooled at room temperature. Using a proSTAR first strand RT-PCR kit (Stratagene, La Jolla, CA, USA), reverse transcriptase and dNTPs were added to the RNA-Oligo (dT) mixture and the reaction was performed at 42°C for 1 h. Each single-strand cDNA was used for subsequent PCR amplification of semaphorin 5A, plexin B3 and β-actin with the latter used as a quantitative control. PCR was carried out in a reaction volume of 25 μL under the following conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 55°C for 50 s, at 72°C for 40 s, and a final extension at 72°C for 5 min on an authorized thermal cycler. The primer sequences used amplification are 5’-CTCAGTCGGTGGAGGC TTAT-3’ and 5’-CAGATTTGAGCCGCAATA-3’ for semaphorin 5A, 5’-TCTCGTCGTGCGGTTCTG-3’ and 5’-CCTTCACCACCCTGCTTGAG-3’ for plexin B3, 5’-CGCAACTGCGATTGTCAT-3’ and 5’-TTC TCTTCGTATGTCAGCACC-3’ for β-actin, respectively. The primer sequences were synthesized by Beijing Genomics Institute (China). The PCR products were resolved in 1.5 % agarose gels and visualized by staining with ethidium bromide. To quantify the PCR products, bands representing the amplified products were analyzed by Quantity One Analysis Software (BIO-RAD Co., USA).

**Real-time PCR**

The reaction mixture volume was made up to 50 μL. Quantitative RT-PCR was performed using SYBR GreenER qPCR SuperMix reagents (Invitrogen) and a Bio-Rad iCycler. Relative transcript quantities were calculated using the ΔΔCt method with β-actin as the endogenous reference gene amplified from the samples. PCR conditions were as follows: an initial melting step at 95°C for 1 min followed by 35 cycles at 95°C for 90 s, at 60°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 10 min. The primers used for RT-PCR are 5’-GGTACTTGTCTTGACGCACCCGCTG-3’ and 5’- ATACTTGGGTTCTGGGTGTG-3’ for semaphorin 5A, 5’-AAAGCCACCGAGGGAGTGGG-3’ and 5’-ACTTGGACGGATGGGAGTGG-3’ for plexin B3, 5’-TGCAGGTGACATCCGAAGG-3’ and 5’-CCTGGA AGGTGGACAGCGGAGG-3’ for β-actin, respectively.

**Western blotting**

Frozen specimens were homogenized in a lysis buffer [50 mMol/L Tris-HCl (pH 7.5), 150 mMol/L NaCl, 1 mMol/L EDTA, 0.25 % sodium deoxycholate, 1 % Triton X-100, 0.1 % sodium dodecyl sulfate (SDS), 1 mMol/L NaF, 1 mMol/L NaVO₄, and protease inhibitors (10 mg/L aprotinin and 1 mMol/L phenylmethylsulfonyl fluoride) were added to obtain total protein. An equal amount of protein, quantified with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA), was subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5 % nonfat milk in Tris buffered saline with Tween 20 [TBST, 50 mMol/L Tris-HCl (pH 7.6), 150 mMol/L NaCl, 0.1 % Tween 20] for 2 h at room temperature, and subsequently incubated with primary anti-rabbit polyclonal antibody (anti-semaphorin 5A diluted at 1:400 and plexin B3 diluted at 1:500 and β-actin diluted at 1:2000 were purchased from Santa Cruz Biotechnology) in a blocking buffer at 4°C overnight. Following a
washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (1:1000, Dako, Glostrup, Denmark) for 2 h at room temperature. The membranes were washed with TBST, and protein bands were visualized with enhanced chemiluminescence according to its manufacturer’s instructions (KPL, Gaithersburg, USA). β-actin bands were taken as a loading control. Protein quantity was analyzed using the UTHSCSA Image Tool 3.0. Target protein expression was evaluated using the relative intensity ratio of target protein/loading control.

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between different groups were assessed by ANOVA using SPSS12.0 statistical software. *P* < 0.05 was considered statistically significant.

RESULTS

**Semaphorin 5A and plexin B3 mRNA expression**

To infer the status of semaphorin 5A and plexin B3 in the invasion and metastasis of gastric carcinogenesis, we evaluated the mRNA expression of semaphorin 5A and plexin B3 using semi-quantitative RT-PCR in 48 samples of primary gastric carcinoma tissue and its corresponding non-neoplastic mucosa as well as matched regional lymph node metastasis. A representative result of RT-PCR for semaphorin 5A and plexin B3 expression in 48 samples of primary gastric carcinoma (Ca) and its corresponding nonneoplastic mucosa (M) as well as matched regional lymph node metastasis (L) examined by RT-PCR. The expression of β-actin was used as an internal control; B: Real time RT-PCR for relative expression levels of semaphorin 5A and plexin B3 in 48 samples of primary gastric carcinoma (Ca) and its corresponding nonneoplastic mucosa (M) as well as matched regional lymph node metastasis (L).

**DISCUSSION**

Semaphorin 5A is a member of class 5 semaphorins which are anchored to cell membranes and characterized by seven type 1 thrombospondin repeats. Plexin B3,
expression increases significantly with gastric carcinoma progression, and semaphorin 5A and plexin B3 may be involved in the processes of gastric cancer invasion and metastasis. Therefore, the novel expression and function of semaphorin 5A and plexin B3 outside of the nervous system not only add more knowledge about semaphorin 5A and plexin B3, but also shed some lights on the pathogenesis of gastric carcinoma, and probably represent a new therapeutic target for gastric carcinoma.

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REFERENCES

1. Gherrardi E, Love CA, Esnouf RM, Jones EY. The sema domain. Curr Opin Struct Biol 2004; 14: 669-678
2. Yazdani U, Terman JR. The semaphorins. Genome Biol 2006; 7: 211
3. Luo Y, Raible D, Raper JA. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 1993; 75: 217-227
4. Negishi M, Oinuma I, Katoh H. Plexins: axon guidance and signal transduction. Cell Mol Life Sci 2005; 62: 1363-1371
5. Kruger RP, Auranndt J, Guan KL. Semaphorins command cells to move. Nat Rev Mol Cell Biol 2005; 6: 789-800
6. Pioton VA, Roche J, Drabkin HA. Semaphorins and their receptors in lung cancer. Cancer Lett 2009; 273: 1-14
7. Sun Q, Nawabi-Ghasimi F, Basile JR. Semaphorins in vascular development and head and neck squamous cell carcinoma-induced angiogenesis. Oral Oncol 2008; 44: 523-531
8. Neufeld G, Kessler O. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nat Rev
Roth L, Koncina E, Satkauskas S, Crémel G, Aunis D, Bagnard D. The many faces of semaphorins: from development to pathology. Cell Mol Life Sci 2009; 66: 649-666

Artigiani S, Conrotto P, Fazzari P, Gilestro GF, Barberis D, Giordano S, Comoglio PM, Tamagnone L. Plexin-B3 is a functional receptor for semaphorin 5A. EMBO Rep 2004; 5: 710-714

Kantor DB, Chivatakarn O, Peer KL, Oster SF, Inatani M, Hansen MJ, Flanagan JG, Yamaguchi Y, Sretavan DW, Giger RJ, Kolodkin AL. Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. Neuron 2004; 44: 961-975

Goldberg JL, Vargas ME, Wang JT, Mandemakers W, Oster SF, Sretavan DW, Barres BA. An oligodendrocyte lineage-specific semaphorin, Sema5A, inhibits axon growth by retinal ganglion cells. J Neurosci 2004; 24: 4989-4999

Woodhouse EC, Fisher A, Bandle RW, Bryant-Greenwood B, Charboneau L, Petricoin EF 3rd, Liotta LA. Drosophila screening model for metastasis: Semaphorin 5c is required for l(2)gl cancer phenotype. Proc Natl Acad Sci USA 2003; 100: 11463-11468

Conrotto P, Corso S, Gamberini S, Comoglio PM, Giordano S. Interplay between scatter factor receptors and B plexins controls invasive growth. Oncogene 2004; 23: 5131-5137

Giordano S, Corso S, Conrotto P, Artigiani S, Gilestro G, Barbets D, Tamagnone L, Comoglio PM. The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol 2002; 4: 720-724

Neufeld G, Shraga-Heled N, Lange T, Guttmann-Raviv N, Herzog Y, Kessler O. Semaphorins in cancer. Front Biosci 2005; 10: 751-760