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Original Articles

Neuropilin-2 mediates lymphangiogenesis of colorectal carcinoma via a VEGFC/VEGFR3 independent signaling

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ABSTRACT

Lymphangiogenesis critically contributes to the lymphatic metastasis of colorectal carcinomas (CRCs), but the underlying mechanism of CRC lymphangiogenesis remains largely elusive. We have previously demonstrated that Semaphorin-3F (SEMA3F) is critically involved in CRC metastasis, and the receptor of SEMA3F, neuropilin-2 (NRP2), originally described as an axon guiding chemorepulsant implicated in nerve development, has been suggested in promoting lymphangiogenesis via acting as an obligate co-receptor of VEGFR3 cooperatively enhancing the activity of VEGF-C. Our present study revealed that in colorectal carcinomas, NRP2 expression levels of tumor-associated lymphatic endothelial cells (LECs) are significantly correlated with the density of tumor lymphatic vessels. *In vitro*, activation of NRP2 in LECs substantially facilitates their migration, sprouting, and tubulogenesis capacity via regulating the rearrangement of cytoskeleton polarity. *In vivo* model further showed that in the xenografts generated from SEMA3F knockdown CRC cells, NRP2 is substantially activated in tumor-associated LECs, resulting in a significantly increased tumor lymphangiogenesis. Further evidence demonstrated that CRC cell induces the activation of NRP2 in LECs to promote tumor lymphangiogenesis via integrinα9β1/FAK/Erk pathway independent VEGF-C/VEGFR3 signaling. Our study for the first time revealed the novel molecular mechanism of NRP2-mediated-lymphangiogenesis in CRCs, suggesting NRP2 as a potential therapeutic target in preventing lymphatic metastasis of CRCs.

Introduction

Metastasis of colorectal carcinoma (CRC), which commonly uses lymphatics, indicates a poor prognosis [1,2]. Tumor neolymphangiogenesis critically contributes to the lymphatic metastasis of CRCs [3–5], but the underlying mechanism attributable to CRC lymphangiogenesis remains largely elusive. It has been reported that tumor lymphangiogenesis is a multiple-factorial process attributed to the interactions between endothelial cells, tumor cells, and other components in the tumor microenvironment. Vascular endothelial growth factor C (VEGF-C) [1,2] is a well known secreted factor inducing tumor lymphangiogenesis [6,7] via interacting with tyrosine kinase VEGF receptor 3 (VEGFR3) on the lymphatic endothelial cells. Although anti-VEGF targeted therapies for CRC patients have led to an improvement in efficacy, the overall outcomes have not met expectations. The relatively disappointing performance of anti-VEGF therapies in the clinic strongly suggests that some other mechanisms of tumor vasculogenesis are worthy of being revealed.

Neuropilin-2 (NRP2) has recently been implicated in lymphangiogenesis. It was reported that NRP2 knockout mice are deficient of small lymphyatic vessels and lymphatic capillaries [8]. Although the precise mechanism for NRP2-induced-lymphangiogenesis remains a matter of debate, most studies suggest that NRP2 acts as an obligate co-receptor of VEGFR3 on lymphatic endothelial cells (LECs) cooperatively enhancing the activity of VEGF-C [9,10]. Intriguingly, our previous study has demonstrated that loss of Semaphorin-3F (SEMA3F), the inhibitory ligand of NRP2, critically contributes to CRC metastasis [11]. We then hypothesize that NRP2 signaling critically contributes to CRC lymphangiogenesis to achieve the full effect of SEMA3F deficiency in CRC metastasis.

To test this hypothesis, in this study, we examined the role of NRP2 in the tumor lymphangiogenesis of CRCs. We demonstrated that NRP2 plays a crucial role in mediating CRC lymphangiogenesis. NRP2 activation in tumor-associated-lymphatic endothelial cells (LECs) significantly promotes their migration and tubulogenesis capacity via the integrinα9β1/FAK/Erk pathway independent of VEGF-C/VEGFR3 signaling. Our findings for the first time revealed a novel

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role and the underlying mechanism of NRP2 involved in CRC pathogenesis.

Materials and methods

Tissue samples

200 tissue specimens used for primary cell isolation, immunohistochemistry, immunofluorescence microscopy and western blot studies were collected from CRC patients who had undergone surgeries at Southwest Hospital, the Third Military Medical University. Tumors were staged by anatomic pathologists in the Department of Pathology, Southwest Hospital, according to the Union for International Cancer Control (UICC) classification system. Half of each fresh tissue sample was used for primary cell isolation, a piece of each specimen was snap-frozen in liquid nitrogen, then stored at –80 °C ultra-freezers for immunofluorescence staining, mRNA and protein isolation, and the rest was fixed in 10% formaldehyde and embedded in paraffin for histological sections. All human experiments were carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association, and were approved by the Ethics Committee of Southwest Hospital, the Third Military Medical University.

Isolation of endothelial cells from human tumors

0.5–3 g pieces of fresh CRC specimen were cut from the border of the malignancy to be used for the isolation of tumor lymphatic endothelial cells (LEC). The specimen was washed by transferring it using sterile forceps from a 50 mL falcon with fresh ice-cold 1× HBSS to a new falcon with 1× HBSS four times consecutively. The specimen was removed from the falcon tube and was placed in a 10-cm cell culture dish to be minced into approximately 10-mm^3 pieces using a fresh, sterile scalpel. Minced tissue were put into a 15-mL falcon tube filled with 3 mL EBM-2-MV (Lonza, Cologne, Germany) supplemented with 0.5% FBS. Collagenase II (50 mL enzyme per 0.1 g tissue; 17,100 U/g) was pipetted into each falcon tube, and EBM-2-MV was added up to a total volume of 5 mL for each falcon. The falcon tubes were then put in the Dynal sample mixer (Invitrogen, Karlsruhe, Germany) at 37 °C for 1 h at 5% CO_2 with the lowest speed available. A 100 mm cell strainer (BD Biosciences) was put on a 50 mL falcon tube and the digested tissue was poured through the strainer. The filter was washed from the inside and outside using 3 mL EBM-2-MV each time, and was centrifuged with 500× *g* for 5 min at 20 °C to discard the supernatant. The cell pellet was resuspended in 5 mL EBM-2-MV, and then was cultivated until 70–80% confluence in a T-25 cell culture flask precoated with 1.5% gelatin for at least 2 h. The isolated cells can be positively selected for VEGFR3 by MACS (mouse-anti-human VEGFR3 antibody was purchased from Chemicon, and was ligated to the MACS obtained from Pierce) after the cultures reach 70–80% confluence. MACS selection was repeated until all nonendothelial cells were removed. Contamination with nonendothelial cells was analyzed by staining an aliquot of the cells for VEGFR3, and subsequent immunocytochemical or FACS analysis. The first confluent T-25 flask of pure LECs is designated as passage 0. One passage is defined by a split ratio of 1:4.

Cell culture

The human colon cancer cell lines SW480 and SW620 were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in L-15 (Invitrogen Corp.) supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. Freshly isolated lymphatic endothelial cells were cultured in ATCC-formulated of F-12K Medium with 0.1 mg/mL heparin, 0.03–0.05 mg/mL ECGS, supplemented with fetal bovine serum at 37 °C under 5% CO_2.

Antibodies and reagents

A monoclonal anti-focal adhesion kinase (FAK) antibody was obtained from Upstate Biotechnology (Upstate, Waltham, MA). A polyclonal anti-phospho-FAK (phospho-Tyr397) antibody, antiphospho-c-Src (Y416), was purchased from BD Biosciences (San Diego, CA). A rabbit monoclonal anti-phospho-Akt, a rabbit polyclonal anti-Akt antibody, a rabbit monoclonal anti-Phospho-p44/42 MAPK (Erk1/2) and a mouse monoclonal anti-Erk antibody were purchased from Cell Signaling (Beverly, MA). Antibody against NRP2 (sc-13117) was purchased from Santa Cruz. Antibodies against integrin α2β1, integrin α4β1, integrinα9β1, integrin α9 and isotype control antibody (mouse IgG1) for these antibodies were purchased from Abcam Inc. A mouse monoclonal antibody against integrin β1 was purchased from Chemicon (Temecula, CA). Phalloidin (TRITC) was purchased from Sigma. A monoclonal anti-GAPDH antibody for Western blotting was purchased from Cell Signaling. A monoclonal antibody of anti-c-Src was from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemical and immunofluorescence microscopy studies

All tissue chip slides were routinely dewaxed, rehydrated, and prepared for immunohistochemistry. The slides were incubated in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase. Antigens were retrieved with 10 mmol/L sodium citrate (pH = 6) for 15 min in a microwave oven. The slides were then incubated with the selected antibody at 37 °C for 1 h and at 4 °C overnight. Slides without treatment with the primary antibody served as negative controls. The slides were developed with an EnVisionTM method (DAKO, Capinteria, CA). The slides were visualized using the diaminobenzidine solution (DAKO, Capinteria, CA), and then lightly counterstained with hematoxylin. Immunohistochemical staining was scored from 0 to 4 as follows. No staining = 0; weak staining = 1; strong staining of 25% or moderate staining of <80% tumor cells = 2; strong staining of 25–50% or moderate staining of >80% tumor cells = 3; strong staining of >50% tumor cells = 4. Meanwhile, Scores 0–1 as negative (recorded as –) and 2–4 as positive (2 recorded as +, 3 as ++, 4 as +++). Ten representative areas were counted from high power fields for each tissue section. Slides were examined and scored independently by 3 pathologists blinded to other patient information.

Samples for immunofluorescence staining were fixed in ice–acetone for 20 min, washed with PBS 3 times for 5 min each, and incubated for 30 min at room temperature in a protein-blocking solution. The sections were incubated with the primary antibodies for 1 h at 37 °C and then at 4 °C overnight. After washing, the sections were incubated at 37 °C for 1 h with appropriate secondary antibodies, including FITC-conjugated goat anti-rabbit IgG (1:50, Santa Cruz), FITC-conjugated goat anti-mouse IgG (1:50, Santa Cruz), or TRITC-conjugated goat anti-mouse IgG (1:50, Beyotime, China). The sections were counterstained with Hoechst 33258 to reveal cell nuclei.

Transfection plasmid information and establishment of stable cell lines

FAK shRNA and control plasmids were obtained and used as previously described [12]. Rac1 siRNA and control plasmids were purchased from Santa Cruz. The VEGFR3 expression plasmid (RC214285) and RNAi plasmid (TG320362) were purchased from Origene. All resultant constructs were verified by DNA sequencing, and then transfected into target cells with lipofectamineTM 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Transfected cells were enriched by selection for 1 week with antibiotics selection. The pBMN-COS-NRP2 vector (Invitrogen Corp) was kindly provided by David Ginty, and used as described previously [11].

Protein extraction and Western blotting

Cell lysates were prepared with M-PER Mammalian Protein Extraction Reagent (PIERCE, PA, USA). A total of 30 μg of lysate proteins were separated by SDS-PAGE after heat denaturation, transferred onto PVDF membranes, and incubated with 5% non-fat milk dissolved in PBS-Tween 20 solution for 1 h, followed by incubation with a primary antibody overnight at 4 °C. After washing, the membranes were incubated with an appropriate HRP-conjugated secondary antibody, and then developed with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biosciences).

Co-immunoprecipitation assay

Total protein lysates (500 μg) from each sample were immunoprecipitated in 400 μL lysate buffer containing 2 μL anti-integrin α9 antibody and inhibitors of various proteases, phosphotases and kinases at 4 °C for 4 h with rotation. Protein A-conjugated agarose beads (25 μL) were then added into the immunoprecipitation reaction with an additional 5 h of rotation at 4 °C. The antigen–antibody complexes were precipitated by a quick centrifugation, followed by 4 times of wash with cold PBS. Controls included an aliquot of rabbit serum to replace the integrin α9 antibody, in the immunoprecipitation reaction. The pellets were suspended in 20 μL of 2× SDS reducing Western blot loading buffer and boiled for 10 min, followed by SDS-PAGE. The integrin α9-immunoprecipitates were subjected to Western blot assay to detect integrin α9 and integrin β1 in the immunoprecipitates. Rac1 activity assay was measured as described in refs. 13 and 14.

ELISA

VEGFC concentrations in the culture medium were quantified using a VEGFC Human ELISA Kit (ab100664) purchased from Abcam.

Quantitative real-time PCR (qPCR)

Total RNAs were isolated using a Peqgold Total RNA Kit including DNase digestion (Peqlab, Erlangen, Germany). RNAs were transcribed into cDNAs using Omniscript (Qiagen, Hilden, Germany). qPCR was performed using the 7900HT Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Expression levels were normalized to β-actin. Reactions were done in duplicate using Applied Biosystems Taqman Gene Expression Assays and Universal PCR Master Mix. The relative expression was calculated by the $2^{(-DDCt)}$ method. The primers are available upon request.

Transwell assay

The migration ability of cells was assessed using Transwell chambers with polycarbonate membrane filters with 24-well inserts (6.5 mm diameter and 8 μm pore