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Original Article

Neuropilin 2 could promote gastric adenocarcinoma lymphatic invasionwith VEGF-C stimulation

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Abstract: Aim: To explore the clinical significance of Neuropilin 2 (NRP2) and vascular endothelial growth factor-C (VEGF-C) in gastric adenocarcinoma. Method: We detected the expression of VEGF-C and NRP2 in 92 patients with gastric adenocarcinoma with immunohistochemistry (IHC) and analyzed the correlation between VEGF-C, NRP2 expression and clinicopathologic factors. With RT-PCR, we analyzed the mRNA level of VEGF-C and NRP2 in tumor tissue and corresponding tissue, tumor in situ and invaded lymph nodes. With transwell assay, we estimated the role of VEGF-C and NRP2 in cell invasion of gastric adenocarcinoma. Results: The high-expression rates of VEGF-C, NRP2 and VEGF-C&NRP2 were 52.17%, 33.70% and 13.04%, respectively. VEGF-C&NRP2 high expression was significantly associated with positive lymph node metastasis (P=0.009). The mRNA level of NRP2 in tumor tissue was significantly higher than that in adjacent tumor tissue (P=0.030), and mRNA levels of both NRP2 and VEGF-C in invaded lymph nodeswereremarkably higher than those in the gastric adenocarcinoma in situ (P=0.020 and 0.010, respectively). Human recombinant VEGF-C at 10 ng/ml could promote invasion of gastric adenocarcinoma cells, and this effect was significantly reduced after NRP2 knockdown, indicating NRP2 was required in VEGF-C induced gastric adenocarcinoma invasion. Conclusion: High expression of VEGF-C&NRP2 was significantly associated with positive lymphatic invasion of gastric adenocarcinoma. VEGF-C could promote the gastric adenocarcinoma cell invasion via stimulating NRP2, indicating that VEGF-C-NRP2 signaling pathway could be a potential blocking target to reduce the lymphatic invasion.

Keywords: VEGF-C, Neuropilin 2, lymphatic invasion, progression

Introduction

As the fourth most common malignancy and the second leading cause of cancer-related death worldwide, gastric cancer affectsabout 989,000 patients annually (7.8% of all cancers) [1]. Gastric cancer is featured with early lymphatic invasion and metastases, as well as the high incidence of recurrence. In Western countries, patients are usually at advanced clinical stages when diagnosed as gastric cancer, which mostly had tumor lymphatic invasion and a poor prognosis with 5-year survival rate less than 30% [2]. The most effective and only curative treatment for gastric cancer is surgery, and the surgical clearance of invaded lymphatic nodes is essential for curative treatment [3]. However, even after curative resection, recurrence is noted in more than half of the cases of advanced-stage disease, which resulted in the poor prognosis of gastric cancer [3]. Histopathologically, gastric cancer mainly refers to gastric adenocarcinoma, which originates from glandular epithelium of the gastric mucosa and overwhelmingly accounts for 90% of gastric cancer [4, 5]. So the exploration of new adjuvant therapies on gastric cancer is mainly focused on gastric adenocarcinoma. Thanks to decades of progresses made in gastric cancer treatment, the overall survival rate issignificantly elevated, but still not satisfactory. This poor outcome promptsenormousefforts to explore new approaches for treatment, especially the targeted therapy. Therefore, much attention hasbeen given to the exploration of new biomarkers and drug targets.

Neuropilin (NRP) is a transmembrane glycoprotein which was first identified as a receptor for the axon semaphorins [3]. NRP family consist of

two members, NRP1 and NRP2 in human beings, which both play essential roles in angiogenesis, axon guidance, cell survival, migration, and invasion [6]. NRP1 and NRP2 are both co-receptors ofvascular endothelial growth factor (VEGF) and semaphorin family members. However, NRP1-/- mice exhibited early embryonic mortality with obvious cardiovascular defect, while NRP2-/- mice displayed ectopic development of peripheral lymph vessels, indicating that NRP1 and NRP2 may play essential role in the development of vascular and lymphatic systems, respectively. Ectopic NRP2 expression has been demonstrated to be associated with the progression in lung cancer, neuroblastoma, pancreatic cancer, osteosarcoma, and bladder cancer, etc [7-10]. NRP2 function in gastric adenocarcinoma was sporadically reported before with experiments in vitro. NRP2 ishigh expressed in human gastric cancer specimens and in gastric cells in vitroand can influencesurvivalin gastro-intestinal cancer cell lines. However, the clinical significance of NRP2 and VEGF-NRP2 signaling pathway in gastric cancer is still undefined.

In our study, we detected the expression of VEGF-C and NRP2 in 92 patients with gastric adenocarcinoma with immunohistochemistry (IHC) and analyzed the correlation between VEGF-C, NRP2 expression and clinicopathologic factors. With RT-PCR, we analyzed the mRNA level of VEGF-C and NRP2 in tumor tissue and corresponding tissue, tumor in situ and invaded lymph nodes. With experiments in vitro, we estimated the expression of VEGF-C and NRP2 in gastric adenocarcinoma cell lines by immunoblotting and the role of VEGF-C and NRP2 in cell invasion of gastric adenocarcinoma by tranwell assay.

Wedetected the expression of VEGF-C and NRP2 with immunoblotting and RT-PCR in gastric adenocarcinoma tissues and with immunoblotting in gastric adenocarcinoma cell lines. As the result, we demonstrated that high expression of VEGF-C&NRP2 was significantly associated with positive lymphatic invasion of gastric adenocarcinoma. VEGF-C could promote the gastric adenocarcinoma cell invasion via stimulating NRP2, indicating that VEGF-C-NRP2 signaling pathway could be a potential blocking target to reduce the lymphatic invasion.

Patients and methods

Patents and follow-up

From 2006 to 2010, a total of 325 patients were diagnosed as gastric adenocarcinoma and underwent surgical operation in Yuhuangding Hosptial of Yantai City and Qilu Hospital of Jinan City, in which 273 patients had available tissue samples, consisting of the primary cohort of our study. In the primary cohort of 273 cases, 92 patients were selected into validation cohort according to the criteria: (1) no severe complications in hospital, (2) approval of experiments of tumor samples from the patients or their relatives, (3) detailed hospital and surgical records. The diagnosis as gastric adenocarcinoma was double confirmed by two senior pathologists. The validation cohort of 92 patients comprised of 56 males and 36 females, with average follow-up 22.5 months.Moreover, 15 pairs of gastric adenocarcinoma samples and corresponding adjacent tumor samples were obtained during surgery and preserved in liquid nitrogen, which were for the mRNA extraction later. All the samples (paraffin-bedded or frozen) were obtained after prior patient consent and approval of the Institutional Clinical Ethics Review Board. The tumor TNM stage was identified according to the guideline of 7th American Joint Committee on Cancer/Union for International Cancer Control.

Cells and reagents

Well differentiated adenocarcinoma cell linnes MKN-7 and MKN-28 were purchased from RIKEN Bioresource Center (Koyadai, Japan), and poorly differentiated adenocarcinoma cell lines SNU-1 and SNU-16 were from American Type Culture Collection (ATCC) (Manassas, USA). All above cell lines were cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/mL streptomycin (HyClone, USA) in 5% CO_2 resuscitation. All used antibodies were purchased from Santa Cruz Corporation (Austin, TX, USA) and all reagents were purchased from Sigma-Aldrich Corporation without special instruction. NRP2 siRNA (sc-36040) and control scrambled RNA were acquired from Santa Cruz (Austin, TX, USA). Lipofectamine 2000 (Invitrogen Com-

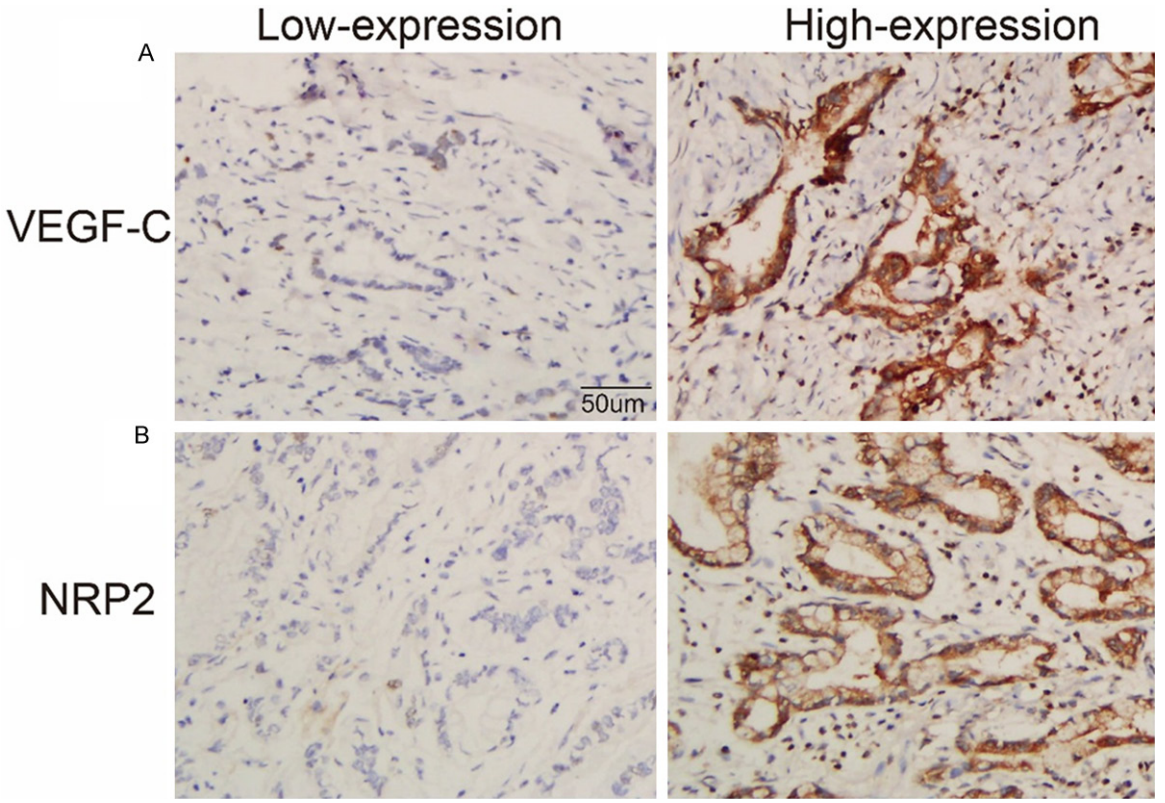

Figure 1. Expression of VEGF-C and NRP2 was detected in gastric adenocarcinoma tissues by IHC. Representative figures for immunohistochemical low and high expression of VEGF-C (A) and NRP2 (B).

pany) was used for transfection according to the manual. Human recombinant VEGF-C was purchased from PeproTech Inc (Rocky Hill, NJ, United States).

Immunohistochemistry and score

Immunohistochemistry (IHC) was used to detect the candidate protein expression with streptavidin peroxidase complex method. Briefly, samples were first deparaffinized with xylene, then incubated in 3% hydrogen peroxide for 10 minutes for inactivation of endogenous peroxidase, followed by incubation in citrate buffer (pH 6.0) and boiled by microwave oven heating for antigen retrieval. Nonspecific bindingwas achieved by soaking in Phosphate Buffered Saline with 5% BSA. Slides were incubated in primary antibody at the dilution 1:100 at 4°C overnight, and in corresponding secondary antibody at 37°C for 1 to 2 hours. Finally, following application of peroxidase complex reagent, 3, 3'-Diaminobenzidine solution was used to incubate for antigen visualization. Results of IHC were evaluated and scored by two senior pathologists unaware of the clinical information.

The scores of positive cell percentage were as follows: 0, less than 10% positive cells; 1, 10%-30% positive cells; 2, 30%-50% positive cells; and 3, > 50% positive cells, while the scores of staining intensity weredefined as: 0 for negative staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The total IHC score was calculated as the product of positive cells multiplied by staining intensity, which ranged from 0 to 9. The cut-off was arbitrarily defined as: score ≥ 4 is high expression and score < 4 is low expression.

RNA extraction and real-time PCR analysis

Total RNA was purified from cancer tissue with Trizol reagent as described before [11]. After RNA extraction, cDNA synthesis and quantitative PCR was performed with the StepOnePlus real-time PCR system (Applied Biosystems) in the SYBR Green way according to the manual. Glyceraldehyde 3-phosphate dehydrogenase

Table 1. Characters of the patients

Characters	Number	Percentage
Gender		
Male	56	60.90%
Female	36	39.13%
Age		
< 60	20	21.73%
≥ 60	72	78.26%
Tumor diameter (cm)		
≤ 5	27	29.35%
> 5	65	70.65%
Differentiation		
Well+Moderate	51	55.43%
Poor	41	44.56%
Tumor invasion		
T1	9	9.78%
T2	9	9.78%
T3	33	35.87%
T4	41	44.57%
Lymph node metastasis		
No(N0)	21	22.82%
Yes(N1/2/3)	71	77.17%
Distant metastasis		
M0	80	86.96%
M1	12	13.04%
TNM stage		
I	10	10.87%
II	26	28.26%
III	44	47.83%
IV	12	13.04%
VEGF-C		
low	44	47.82%
high	48	52.17%
NRP2		
low	61	66.30%
high	31	33.70%
VEGF-C&NRP2		
low	80	86.96%
high	12	13.04%

(GAPDH) was applied as an internal control. The mRNA levels were expressed as normalized ratios (VEGF-C or NRP2 mRNA/G6PDH mRNA). The sequences of primers used for real-time PCR experiments weredesigned following previous studiesand shown below [12, 13].

NRP2 forward: 5'-GGATGGCATTCCACATGTTG-3'; NRP2 reverse: 5'-ACCAGGTAGTAACGCGCAG-AG-3'; VEGF-C forward: 5'-CACGAGCTACCTC-AGCAAGA-3'; VEGF-C reverse: 5'-GCTGCCTG-ACACTGTGGTA-3'; GAPDH forward: 5'-TGGAGA-ATGAGAGGTGGGATG-3'; GAPDH reverse: 5'-GAGCTTCACGTTCTTGTATCTGT-3'.

Immunoblotting

Expression of cellular protein was detected by immunoblotting. Briefly, cytoplasm proteinswereextracted by lysis in the RIPA lysis and centrifuged at 11,000 rpm at 4°C for 20 minutes. Concentration of supernatant containing cellular proteins was detected with BCA detection kit (Beyotime Institute of Biotechnology, Shanghai, China). Total amount of 10 μg protein was electrophoresed in a SDS-PAGE gel, then transferred to PVDF membrane (PALL Company, USA). After incubatedin corresponding primary antibody (1:1000) overnight at 4°C and in secondary antibody labeled with horseradish peroxidase for 2 hours at 37°C, membranes were finally visualized by ECL (Millipore Company).

Invasion assay

Transwell assay was used to evaluate the invasive ability of gastric adenocarcinoma cell lines. Pre-matrigel-coated transwell 24-well plates were purchased from BD Company. Cells were trypsinized and seededinto tranwell upper chamber and cultured for 6 hours for adhesion. Then serum-free medium was changed to starve cells for 6 hours. After that, medium in lower chamber was changed into 10% FBS-containing medium and used for cell culture for 12 hours. Finally, cells were fixed in methanol and stained in Giemsa stain. Cells on upper filter surface were removed using a cotton swab and invasive cells were observed and counted at × 200 magnification in at least five random visual fields. Cell number of control group was set as baseline and number of other group was standardized by ratio to baseline. Analyzed data were from three independent experiments.

Statistical analysis

All data were analyzed with software SPSS 17.0 (IBM cooperation, USA). The correlation between VEGF-C and NRP2 expression and other clinicopathologic parameters were evaluated by Chi-square test. The difference between compared groups was analyzed by Student

Table 2. Correlation between VEGF-C, NRP2 and clinicopathologic parameters

Characters	VEGF-C		P*	NRP2		P*	VEGF-C&NRP2		P*
	Low	High		Low	High		Low	High	
Gender									
Male	28	28	0.602	35	21	0.332	48	8	0.656
Female	16	20		26	10		32	4	
Age									
< 60	7	13	0.191	12	8	0.504	15	5	0.093
≥ 60	37	35		49	23		65	7	
Tumor diameter (cm)									
≤ 5	16	11	0.176	14	13	0.062	23	4	0.748
> 5	28	37		47	18		57	8	
Differentiation									
Well+Moderate	27	24	0.273	30	21	0.088	43	8	0.396
Poor	17	24		31	10		37	4	
Tumor invasion									
T1	3	6	0.694	5	4	0.369	7	2	0.399
T2	4	5		4	5		7	2	
T3	18	15		22	11		31	2	
T4	19	22		30	11		35	6	
Lymph node metastasis									
No (N0)	12	9	0.330	14	7	0.968	21	0	0.009
Yes (N1/2/3)	32	39		47	24		59	12	
Distant metastasis									
M0	39	41	0.646	53	27	0.977	69	11	0.584
M1	5	7		8	4		11	1	
TNM stage									
I	5	5	0.060	4	6	0.344	7	3	0.164
II	18	8		18	8		25	1	
III	16	28		31	13		37	7	
IV	5	7		8	4		11	1	

*Chi-square.

t-test without special note. Column and spot graph was displayed with ± SEM. $P < 0.05$ was considered statistically significant.

Results

Expression of VEGF-C and NRP2 in gastric adenocarcinoma

The expression of VEGF-C and NRP2 was detected by IHC first. As a secreted growth factor, VEGF-C was both observed in the cytoplasm and the membrane (**Figure 1A**), while NRP2 was mostly observed in the membrane of gastric adenocarcinoma as a co-receptor of VEGF (**Figure 1B**). As detailed described in Patients and Methods, expression of VEGF-C and NRP2 was scored, which was referred as the criteria of subgrouping. The validation cohort was further divided into high-expression and low-expression group according to VEGF-C and NRP2 score. In our experiment, the high-expression rates of VEGF-C and NRP2 were 52.17% and 33.70%, respectively. Moreover, we defined a subgroup as high-expression of both VEGF-C and NRP2 (VEGF-C&NRP2) for better description of the role of VEGF-C and NRP2 synergic function. The positive rate of VEGF-C&NRP2 was 13.04% in our study (**Table 1**).

Correlation between VEGF-C, NRP2 and the clinicopathologic factors

The correlation between VEGF-C, NRP2 and the clinicopathologic parameters was analyzed by

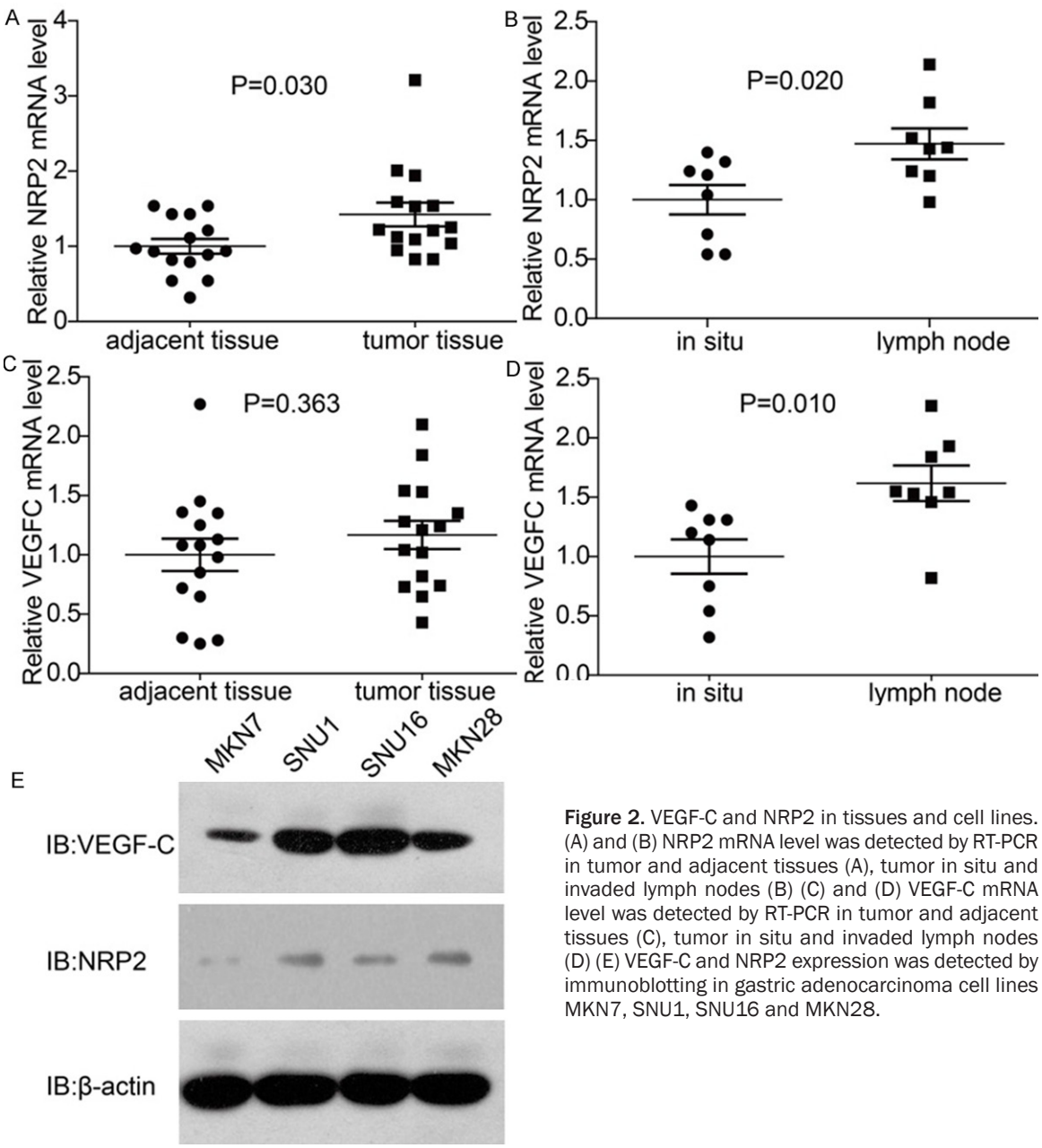

Figure 2. VEGF-C and NRP2 in tissues and cell lines. (A) and (B) NRP2 mRNA level was detected by RT-PCR in tumor and adjacent tissues (A), tumor in situ and invaded lymph nodes (B) (C) and (D) VEGF-C mRNA level was detected by RT-PCR in tumor and adjacent tissues (C), tumor in situ and invaded lymph nodes (D) (E) VEGF-C and NRP2 expression was detected by immunoblotting in gastric adenocarcinoma cell lines MKN7, SNU1, SNU16 and MKN28.

Chi-square test (**Table 2**). Unfortunately, no clinicopathologic factors were significantly associated with separate VEGF-C or NRP2 expression. However, cases of VEGF-C&NRP2 high expression had more cases of positive lymph node metastasis (P=0.009), which indicated that the signaling of VEGF-C and NRP2 may promote the lymphatic invasion of gastric adenocarcinoma.

Expression of VEGF-C and NRP2 in gastric tumor tissues and cell lines

To double confirm the expression of VEGF-C and NRP2 expression in gastric adenocarcinoma, we further detected the mRNA level of VEGF-C and NRP2 in frozen tissues without formalin-fixing and paraffin-bedding, including tumor tissues and adjacent tumor tissues, in situ tumor tissues and invaded lymph nodes. Interestingly, NRP2 high expression was more frequent in tumor tissues rather than that in adjacent tumor tissues (P=0.030), suggesting that NRP2 may play an oncogenic role in gastric adenocarcinoma progression (**Figure 2A**). Moreover, invaded lymph nodes had higher NRP2 mRNA level than tumor in situ, which supported the hypothesis that NRP2 could pro-

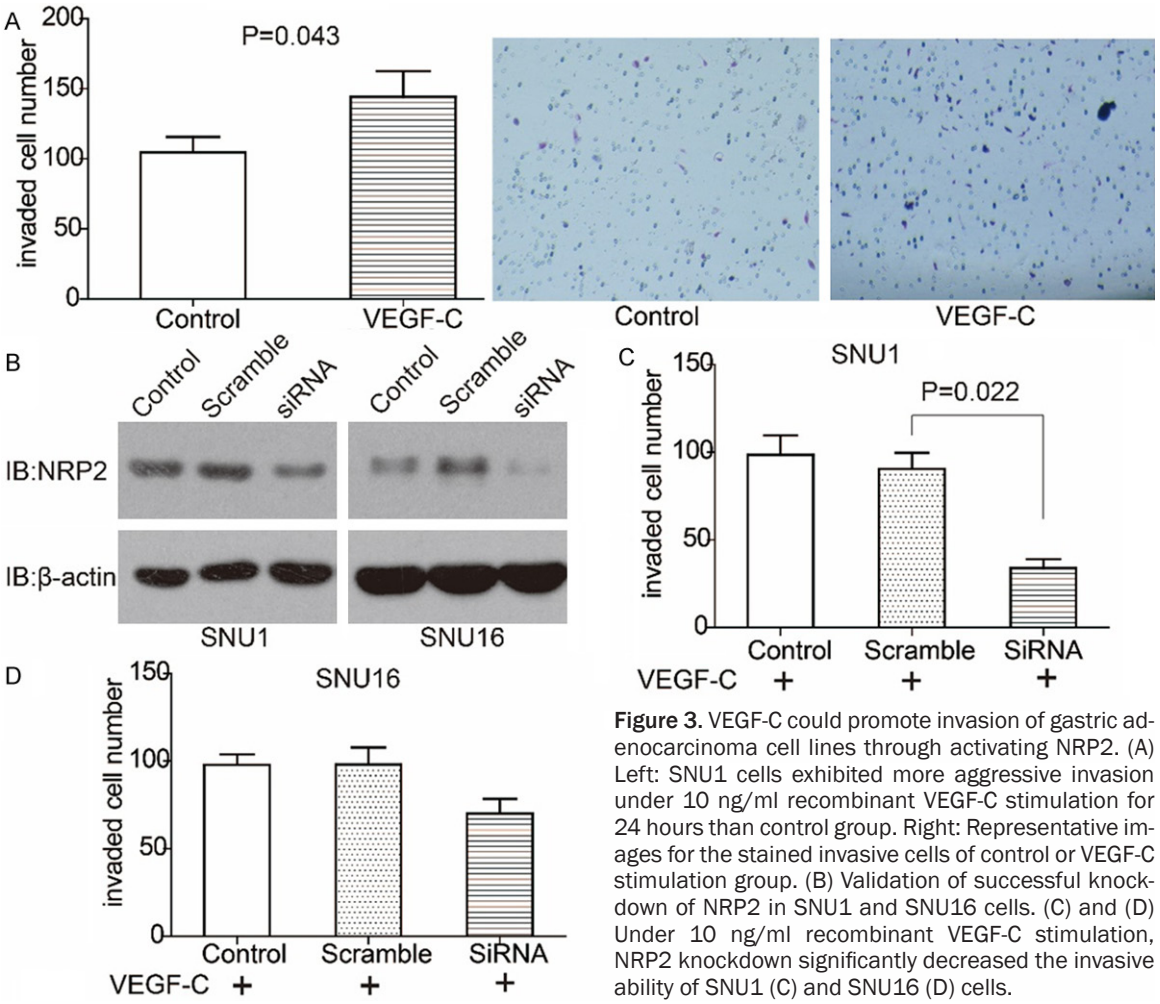

Figure 3. VEGF-C could promote invasion of gastric adenocarcinoma cell lines through activating NRP2. (A) Left: SNU1 cells exhibited more aggressive invasion under 10 ng/ml recombinant VEGF-C stimulation for 24 hours than control group. Right: Representative images for the stained invasive cells of control or VEGF-C stimulation group. (B) Validation of successful knockdown of NRP2 in SNU1 and SNU16 cells. (C) and (D) Under 10 ng/ml recombinant VEGF-C stimulation, NRP2 knockdown significantly decreased the invasive ability of SNU1 (C) and SNU16 (D) cells.

mote the lymphatic invasion of gastric adenocarcinoma (**Figure 2B**). VEGF-C mRNA level in tumor tissues or adjacent tumor tissues had no significant difference (P=0.363) (**Figure 2C**), but NRP2 level in lymph nodes was significantly higher than that in tumor in situ (**Figure 2D**), indicating that NRP2 played an essential role in lymphatic invasion as well. Moreover, four gastric adenocarcinoma cell lines were selected to detect the expression of NRP2 and VEGF-C by immunoblotting, including cell line SUN1, SNU16, MKN7 and MKN28. Expression of NRP2 and VEGF-C was both detectable, but with different abundance. SNU16 had the highest VEGF-C expression, while SNU1 and MKN28 had the similar and highest NRP2 expression (**Figure 2E**).

NRP2 could promote invasion of gastric adenocarcinoma cells under VEGF-C stimulation

We observed that high expression of VEGF-C&NRP2 was significantly associated with positive lymphatic invasion. To further explore the underlying mechanism of this phenotype, we performed experiments in vitro to estimate the NRP2 and VEGF-C role in gastric cell line invasion. Human recombinant VEGF-C at 10 ng/ml was used to stimulate SNU1 cell line for 24 hours in the transwell chamber. It turned out that SNU1 under 10 ng/ml had remarkably more invaded cells than the control group (P=0.043) (**Figure 3A**). To identify whether NRP2 played arole in this VEGF-C-induced invasion, weknocked down the expression of NRP2 with siRNA in SNU1 and SNU16 cell lines. Successful knockdown was validated by immunoblotting (**Figure 3B**). The invasive ability of SNU1 and SNU16 was detected by transwell assay under 10 ng/ml VEGF-C stimulation after NRP2 knockdown. In our experiment, we demonstrated that the invasion induced by VEGF-C stimulation was impaired when NRP2 was knocked down in both SNU1 and SNU16 cells (**Figure 3C** and **3D**), proving that NRP2 was

required in the invasion triggered by VEGF-C activation.

Discussion

Gastric cancer is one of the most lethal threats to human health. In 2012, an estimated 951,600 new stomachcancer casesoccurred, with 723, 100 deaths, and the morbidity and mortality of gastric cancer is still increasing worldwide [14]. Although the development of endoscopy helps to detect the early stages of gastric cancer, most cases are still diagnosed at an advanced stage, which resultsin a poor prognosis [15]. The only curative treatment for localized gastric cancer is surgery, but most patients lose the best opportunity for surgery, and even after curative resection, recurrence is noted in more than half of the cases of advanced-stage disease [1], which could be partially ascribed to the lymphatic invasion of tumor cells. A main problem of treatment to gastric adenocarcinoma is how to restrict the lymphatic invasion, so that more curative surgeriescould be achieved. Although many new therapeutic approaches haveappeared, such as new surgical instruments and new chemical drugs, the 5-year overall survival rate of patients who already had lymphatic invasion still lingered under 30% [2]. So new drugs targeting at key molecules in lymphatic invasion are in urgent need now.

Lymphatic pathway is the most important way for the spread of gastric cancer, but the underlying mechanisms of how lymphatic spreading and the role of lymphangiogenesis (the growth of lymphatics) in gastric metastasis has been less clear. In our study, we found that double high expression of VEGF-C and NRP2 was significantly associated with positive lymph node invasion in gastric adenocarcinoma for the first time, which strongly suggested that VEGF-C and NRP2 could promote the lymphatic invasion of gastric cancer. VEGF-C was the first discovered lymphangiogenic growth factor [16]. VEGFC havehigher affinity for VEGFR2 and VEGFR3 than VEGFR1, which are predominantly expressed inblood vascular and lymphatic endothelia, respectively [17]. It iswell-acknowledged that VEGF-C is able to stimulate the migration of endothelial cells and induce vascular permeability and endothelial-cell proliferation, which was also verified in our experiments. Our finding that the facilitation of lymphatic invasion depended on VEGF-C and NRP2 synergic function may help find the underlying mechanism of why gastric adenocarcinoma had higher probability of lymphatic invasion and help find an effective therapeutic drug for gastric adenocarcinoma.

In our study, we demonstrated that human recombinant VEGF-C could promote gastric adenocarcinoma cells, which required the participation of NRP2. Considering that NRP2 was the co-receptor for VEGF-C, we concluded that VEGF-C triggered the downstream signaling pathway such as Raf-MEK-MAPK pathway and FAK-paxillin pathway by activating VEGFR and NRP2. Moreover, since VEGF-C is a secreted growth factor and is widely expressed in gastric adenocarcinoma cells, we hypothesized that the vulnerable lymph node invasion may be a result of continuous VEGF-C stimulation in a paracrine pathway. This bold hypothesis is certainly required further experiments to validate, but we are convinced that our results could inspire more interest on VEGF-C and NRP2 in gastric lymphatic invasion, which will help verify our hypothesis. Fortunately, there are several available monoclonal antibody drugs targeted at VEGF-VEGFR signaling pathway, such as Bevacizumab, Ramucirumab, Sorafenib etc. However, whether these drugs are effective on gastric cancer treatment needs further clinical investigations and trials.

In summary, we detected the expression of VEGF-C and NRP2 with immunoblotting and RT-PCR in gastric adenocarcinoma tissues and with immunoblotting in gastric adenocarcinoma cell lines. For the first time, we demonstrated that double high-expression of VEGF-C and NRP2 was significantly associated with positive lymphatic invasion. Moreover, we proved that NRP2 and VEGF-C mRNA level in invaded lymph nodeswas remarkably higher than that in tumor in situ, and that NRP2 mRNA in tumor tissues was significantly higher than that in adjacent tumor tissues. With experiments in vitro, we proved that recombinant VEGF-C could promote the invasion of gastric adenocarcinoma cell lines SNU1 and SNU16, and NRP2 was required in this process.

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Disclosure of conflict of interest

None.

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