

Effects of NM-3 on lymphatic vessel density and vascular endothelial growth factor of colon cancer in orthotopic implantation model of a severe combined immune deficiency mice

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Abstract

The molecular mechanisms involved colon cancer tumorigenesis and development of colon cancer remain unclear. The aim of this study is to explore the inhibitive effects of NM-3 on lymphatic vessel density and vascular endothelial growth factor of micrometastatic lesion of orthotopic implanted colon cancer in the severe combined immune deficiency (SCID) nude mice. Human colon cancer SW1116 cells were orthotopically implanted into the colon of the nude mice. Twenty-eight SCID nude mice were randomly divided into four groups (7 mice for each group) after one week feeding and then the nude mice were treated with carboplatin and NM-3 via intraperitoneal injection twice a week for 8 weeks. The mice were sacrificed after 8 weeks and the vascular endothelial growth factor-C (VEGF-C), VEGF-D, VEGF-R-3 and lymphatic vessel density (LVD) were analyzed using immunohistochemistry staining assay. LVD in NM-3 treated mice was significantly lower than that of control (normal saline treated) mice. The expression of VEGF-C, VEGF-D, and VEGF-R-3 and the expression of mRNA of VEGF-C, VEGF-D, and VEGF-R-3 in NM-3 treated mice were significantly lower than that of control mice. The NM-3 inhibited the growth of colon cancer in the SCID mice of orthotopic implantation model, and this effect may be related to the inhibitive effects of NM-3 on the lymphangiogenesis and vascular endothelial growth factor in colon cancer. NM-3 and carboplatin played a synergistic role in inhibiting lymphangiogenesis of human colon cancer in SCID nude mice and the further investigation of molecular mechanisms involved in colon cancer metastasis will provide an important evidence for understanding of lymphangiogenesis of human colon cancer.

Introduction

Colon cancer is one of the most common gastrointestinal malignant tumors and it is one of the leading causes of cancer-related deaths worldwide. In the past five years, it is reported that the incidence of colon cancer in Asia is increased and the prognosis of colon cancer is related to the potential metastasis and lymphangiogenesis of the tumor. The prognosis of advanced colon cancer is very poor. Until now, the molecular mechanisms involved colon cancer tumorigenesis remains unclear.¹

Because of the lack of specific lymphatic endothelial cells (LEC) markers, our previous studies using LMD and P²⁷-based RNA amplification and cDNA microarray could not discriminate between lymphatic and blood vessels.² Recently, some LEC specific markers (VEGF-C, VEGF-D, and VEGF-R-3) were discovered. NM-3 is a novel angiogenesis inhibitor isolated from a culture filtrate of streptovercillium eurocidicum. Previous studies have revealed that NM-3 could inhibit the expression of VEGF family and have direct inhibitory effects on vessel endothelial cells of colon cancer. It is also showed that NM-3 combined with carboplatin can suppressed the growth of human colon cancer SW1116 cells xenografted or orthotopic implanted in the nude mice.³ These results suggested that NM-3 combined with carboplatin could not only increase anticancer effect and induce the apoptosis of human colon cancer cells but also aggravate toxicity to the cancer cells. VEGF-R-3 is one of the specific markers of lymphatic vessels in tumors. Based on this evidence, we investigated whether NM-3 could inhibit lymphangiogenesis in SCID nude mice that orthotopically implanted human colon cancer from SW1116 cells. LVD, subtypes of VEGF, VEGF-C, VEGF-D, VEGF-R-3 and their mRNA were studied using podoplanin and FQ-PCR assays.

Materials and Methods

Chemicals

VEGF-C, VEGF-D, and podoplanin polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA). VEGF-R-3 polyclonal antibody was obtained from AMS Biotechnology (Europe). Colon cancer SW1116 cell line and NM-3 were kindly provided by Professor Robert (New York University, USA).

Animal model

Male severe combined immune deficiency (SCID) nude mice were obtained from Shanghai Experimental Animal Center of

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Chinese Academy of Sciences (SPF, NO.SCXK033). Six to seven week-old and body weight of 20-25 g mice were used. Human colon cancer SW1116 (a poorly differentiated adenocarcinoma) cell line was originally derived from a primary tumor and maintained by passage in the subcutis of nude mice. Animal models were set up via orthotopic implantation of histologically intact tissue of human colon carcinoma. The nude mice were anesthetized with 4.3% trichloraldehyde hydrate. An incision was made through the left upper abdominal pararectal line. Then the peritoneal cavity was carefully exposed and a part of the serosa membrane in the middle of the greater curvature of the stomach was mechanically injured using scissors. A tumor piece was fixed on each injured site of the serosal surface. The stomach was returned to the peritoneal cavity, and then the abdominal wall and skin were closed. After 7 days, the mice were randomly separated into four groups with seven mice per group. Carboplatin (5 mg/kg), NM-3 (10 mg/kg), carboplatin (5 mg/kg) combined with NM-3 (10 mg/kg), or normal saline (control group) were intraperitoneally injected into the each mice group respectively, twice a week. Eight weeks later, the mice were sacrificed by cervical dislocation. Tumors were resected aseptically. Necrotic tissues were cut off and the remaining healthy tumor tissues were minced into pieces (about 2x2 mm in diameter) using scissors and were kept in Hank's balanced salt solution. Each tumor piece was weighed and adjusted to 50 mg, and then the tumor samples were immediately frozen in liquid nitrogen for later use.

Immunohistochemistry staining assay

Immunohistochemical staining of podoplanin, VEGF-C, VEGF-D and VEGF-R-3 was performed using the streptavidin-peroxidase technique. Paraffin embedded tissues were cut into 4 μm thick sections and placed on saline coated slides. After deparaffinization, antigen retrieval was performed by immersing the sections in 10 mmol/L citrate buffer (pH 6.0) and heated twice in a microwave oven (95°C) for 5 minutes each time. Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in distilled water for 10 min. Tissue sections were incubated with normal donkey serum for 20 min at room temperature and then incubated with polyclonal antibodies against LYVE-1, Prox-1, VEGF-C, VEGF-D and podoplanin in normal donkey serum for 2h at room temperature. Bound antibodies were detected with secondary antibody and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. The sections were counterstained with hematoxylin.

Evaluation of immunohistochemical staining

IHC-image analysis was performed using IMS Cell Analysis System (Shanghai Sheng-teng Ltd. China). Every section was analyzed in at least 3 different visual fields ($\times 250$, 2.96V, 2.51A) to assay positive areas, positive percentage and optical density. The expression value was calculated as $0.095 \times$ average of three values.

Lymphatic vessel density counting

After scanning the stained sections of podoplanin at low magnification ($\times 100$), five areas of carcinoma with the greatest number of distinctly highlighted intratumoral lymphatic foci (hot spots) were selected and vessels were counted in a representative high magnification ($\times 200$) field in each of these five areas. Single immunoreactive endothelial cells, or endothelial cell clusters separated from other microvessels were counted as individual microvessels. Endothelial staining in large vessels with tunica media and non-specific staining of nonendothelial structures were disregarded in microvessel counts. Mean visual microvessel density for podoplanin was calculated using the average of five counts.

Fluorescence-based quantitative polymerase chain reaction

Amplification was carried out in a volume of 50 μL mixture, containing 0.5 μL of each primer (25 $\mu\text{mol/L}$), 0.5 μL dNTPs (10 mmol/L), 1 U of Taq DNA polymerase, 10 μL of $5 \times$ PCR buffer (with Mg^{2+} , 25 mmol/L), 5 μL of

cDNA templates and 0.5 μL fluorescent probe, and then made up to a volume of 50 μL with deionized water. The primer sequence of VEGF-C was: sense 5'-GAATTCTCGGTGTGTGACA-3' and antisense: 5'-CTATCTCACAGCCTTCCTG-3'. The primer sequence of VEGF-D was: sense 5'-CCCA-TGAAGCCTTGTATACCA-3' and antisense: 5'-TGGAAGCG-GAACG-GAAACT-3'. The primer sequence of VEGF-R-3 was: sense 5'-ACGGCCT-TGGTGAAGTGGC-3' and antisense: 5'-CGTTGACTCCTCCGTGATGATG-3'. The FQ-PCR amplification was accomplished as the following: an initial denaturation at 93°C for 2 min was followed by 10 cycles of denaturation at 93°C for 45 sec, annealing at 55°C for 60 sec, and extension at 93°C for 45 sec followed by 30 cycles. Finally, an additional extension was achieved for 1 min at 55°C.

Statistical analysis

The results were expressed as mean \pm SD. Statistical analysis was performed using t test by the statistical software SAS version 8. $P < 0.05$ was considered statistically significant.

Table 1. Effects of NM-3/carboplatin on lymphatic vessel density in different groups(mean \pm SD).

Group	N	LVD (%)
Control	7	7.35 \pm 0.55
NM-3	7	4.72 \pm 0.50 ^a
Carboplatin	7	6.34 \pm 0.49
NM-3 plus carboplatin	7	2.17 \pm 0.13 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Table 2. VEGF-C, VEGF-D, VEGF-R-3 levels of orthotopic implanted tumor in different groups (mean \pm SD).

Group	n	VEGF-C (μm^2)	VEGF-D (μm^2)	VEGF-R-3 (μm^2)
Control	7	2962.84 \pm 519.77	1882.15 \pm 359.38	2123.05 \pm 117.99
NM-3	7	2106.01 \pm 437.11 ^a	1032.25 \pm 460.44 ^b	1222.05 \pm 470.80 ^a
Carboplatin	7	2835.14 \pm 509.12	1785.23 \pm 334.27	2104.02 \pm 114.37
NM-3 plus carboplatin	7	1136.07 \pm 216.09 ^b	837.25 \pm 160.28 ^b	760.13 \pm 206.16 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Table 3. Expression of VEGF-C mRNA, VEGF-D mRNA, VEGF-R-3 mRNA of in different groups (mean \pm SD)

Group	n	VEGF-C mRNA	VEGF-D mRNA	VEGF-R-3 mRNA
Control	7	49.18 \pm 3.12	56.26 \pm 4.12	52.13 \pm 3.17
NM-3	7	29.68 \pm 1.49 ^a	30.26 \pm 0.80 ^a	32.42 \pm 1.02 ^a
Carboplatin	7	41.10 \pm 2.91	52.17 \pm 3.89	48.35 \pm 2.97
NM-3 plus carboplatin	7	21.10 \pm 1.95 ^b	19.26 \pm 1.89 ^b	18.40 \pm 1.64 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Results

Lymphatic vessel density count using staining of podoplanin

LVD count by staining of podoplanin, LVD in NM-3 group and NM-3 plus carboplatin group were significantly lower than that of control group (Table 1).

The expression of VEGF subtypes in nude mice

The expression of VEGF-C, VEGF-D, VEGF-R-3, VEGF-C mRNA, VEGF-D mRNA, and VEGF-R-3 mRNA in the NM-3, and NM-3 in combination with carboplatin treated mice was significantly lower compared to that of the control group (Table 2, 3), but there was no significant difference between carboplatin and control group (Table 2, 3) (Figure 1).

Discussion

NM-3 has special anti-tumor effect. NM-3 in combination with carboplatin may play an active synergistical role. However, human colon cancer treated by NM-3 in combination with carboplatin was not reported in the SCID nude mice.³

Up to date, angiogenesis and lymphangiogenesis have been reported to play an important role in metastasis of colon cancer. Some researchers found that VEGF-C over-expression tumor cells could promote metastasis of lymph node and increase tissue LVD.^{4,8} Inhibiting lymphangiogenesis and inducing apoptosis of colon cancer have become one of the new treatments of colon cancer.^{9,12} Specific markers, such as podoplanin, VEGF-C, VEGF-D,

VEGFR-3 and the others, such as Weibel-Palade body PAL-E D2-40 etc. of lymphatic endothelial cells (LEC) could be regarded as the treatment targets. As an inhibitor of angiogenesis, subsequent work has shown that NM-3 exhibits direct cytotoxic effects on endothelial and carcinoma cells. Moreover, NM-3 has been found to potentiate the therapeutic effects of certain chemotherapeutic agents, including 5-fluorouracil, paclitaxel, and cyclophosphamide. Our previous study showed that NM-3 combined with carboplatin could suppress the growth of human colon cancer SW1116 cells *in vivo* and xenografted under abdominal skin of the nude mice, suggesting that NM-3 has complex effects which include inhibiting lymphangiogenesis, inducing apoptosis, increasing anticancer effect and aggravating toxicity in combination with carboplatin. Our previous studies showed the inhibitive effect of NM-3 on orthotopically implanted tumors from human gastric cancer (SGC-7901) cell line in the SCID nude mice.¹ But effects of NM-3 on orthotopic implanted human colon cancer were not reported. The VEGF family consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor (PlGF). VEGF-A acts through VEGFR-1 and -2 receptors, VEGF-C and -D act through VEGFR-2 and VEGFR-3. Both of VEGF-C and VEGF-D are able to induce proliferation and migration of lymphatic endothelial cells *in vitro*. Some researchers studied the expression of VEGF-C and VEGF-D in human tumors and their possible connection to metastasis. The majority of such studies confirmed a positive correlation between growth factor expression and adverse oncological features. VEGF-R-3 was restricted to LECs in the adult. The importance of VEGF-R-3 for the development of the lymphatic vasculature has been shown recently. The correlation of VEGF-C/VEGF-R-3 axis to the lymphatic spread of tumors was documented in several experimental models and clinic pathological studies in a variety of human malignancies. Recently, it was reported that the serum VEGF-C levels might be a useful biomarker in determining the presence of lymph node metastasis and the serum VEGF-C levels correlate with VEGF-C tissue expression in patients with colon cancer. It was also showed that both VEGF-D and VEGF-R-3 were independent prognostic biomarkers for identifying the poor prognosis in patients after resection of the colon adenocarcinomas. Podoplanin was originally found on the surface of rat glomerular epithelial cells (podocytes) and linked to flattening of foot processes (hence the name "podoplanin") that occurs in glomerular diseases. There are studies indicated that podoplanin is regulated by Prox-1. Studies also indicated that endothelial cells expression podoplanin had the same structure as lymphatic endothelial cells, so it is a specific

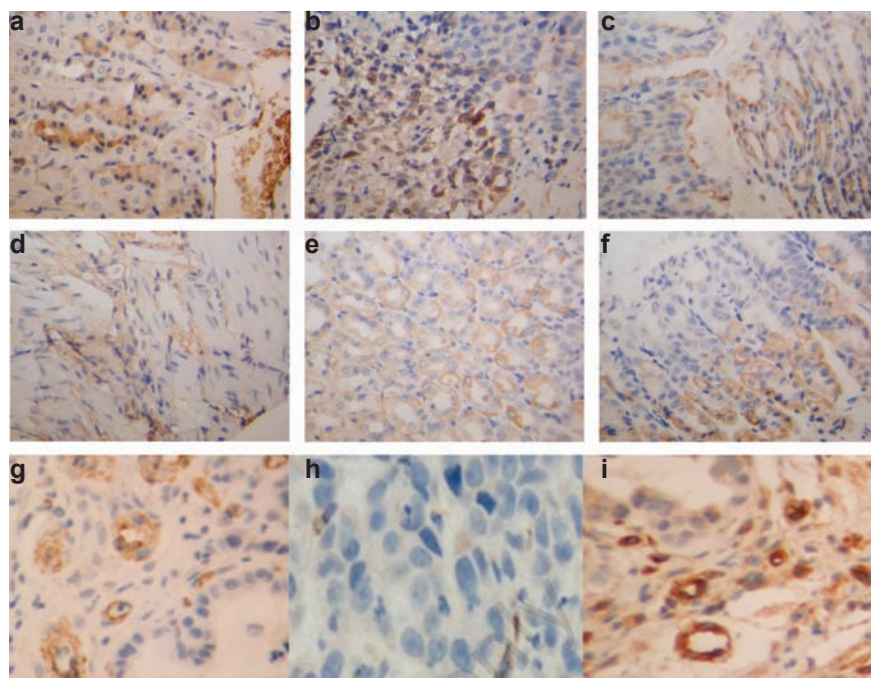


Figure 1. a) Expression of VEGF-C in control group. b) Expression of VEGF-D in control group. c) Expression of VEGF-R-3 in control group. d) Expression of VEGF-C in NM-3 group. e) Expression of VEGF-D in NM-3 group. f) Expression of VEGF-R-3 in NM-3 group. g) Expression of VEGF-C in NM-3 plus carboplatin group. h) Expression of VEGF-D in NM-3 plus carboplatin group. i) Expression of VEGF-R-3 in NM-3 plus carboplatin group.

marker of LECs. Prox1 was also showed a general marker and tumor-associated lymphatic vasculature. In addition, it was also determined that Prox1, VEGF-R-3 and LYVE-1 were expressed similarly in lymphatic endothelial cells of normal adult and tumor tissues. In this study, we found that the expression of VEGF-C, VEGF-D, VEGF-R-3 and their mRNAs were decreased significantly after treatment of NM-3 or NM-3 combined with carboplatin compared with those of control group, and the expression level of prox-1 was significantly different among the NM-3, NM-3 combined with carboplatin and carboplatin groups. The calculating of LVD also showed to be lower than control group after NM-3 or NM-3 and carboplatin treatment, which indicated that NM-3 has the effect of inhibiting lymphangiogenesis but carboplatin has not this function. These results also showed that combined drug use could play a stronger effect than the single drug use in inhibiting the growth of colon cancer cells.¹³⁻¹⁹ In our study, some results had no significant different between the two treatment groups which indicated that the major function of NM-3 may inhibit lymphangiogenesis.²⁰⁻²⁶ Meanwhile, the quantity of models is so small and lack of NM-3 which made the results more valuable than theoretically.²⁷⁻²⁹

In conclusion, our studies suggested that NM-3 and carboplatin played a synergistic role in inhibiting lymphangiogenesis of human

colon cancer in SCID nude mice and the further investigation of molecular mechanisms involved in advanced colon cancer will provide an important evidence for understanding of lymphangiogenesis of human colon cancer.

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