RESEARCH COMMUNICATION

New Model of In-situ Xenograft Lymphangiogenesis by a Human Colonic Adenocarcinoma Cell Line in Nude Mice

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Abstract

Objective: To explore a new model of in-situ xenograft lymphangiogenesis of human colonic adenocarcinomas in nude mice. Method: On the basis of establishing subcutaneous xenograft lymphangiogenesis model of human colonic adenocarcinoms, in-situ xenografts were established through the in situ growth of the HT-29 human colonic adenocarcinoma cell line in nude mice. The numbers of lymphangiogenic microvessels, the expression of lymphatic endothelial cell markers lymphatic vessel endothelial hyaloronic acid receptor-1 (LYVE-1), D2-40 and the lymphatic endothelial growth factors vascular endothelial growth factor-C (VEGF-C), -D (VEGF-D) and receptor-3 (VEGFR-3) were compared by immunohistochemical staining, Western bolt and quantitative RT-PCR in xenograft in-situ models. Results: Some microlymphatics with thin walls, large and irregular or collapsed cavities and increased LMVD, with strong positive of LYVE-1, D2-40 in immunohistochemistry, were observed, identical with the morphological characteristics of lymphatic vessels and capillaries. Expression of LYVE-1 and D2-40 proteins and mRNAs were significantly higher in xenografts in-situ than in the negative control group (both P<0.01). Moreover, the expression of VEGF-C, VEGF-D and VEGFR-3 proteins and mRNAs were significantly higher in xenografts in-situ (both P<0.01), in conformity with the signal regulation of the VEGF-C,-D/VEGFR-3 axis of tumor lymphangiogenesis. Conclusions: In-situ xenografts of a human colonic adenocarcinoma cell line demonstrate tumor lymphangiogenesis. This novel in-situ animal model should be useful for further studying mechanisms of lymph node metastasis, drug intervention and anti-metastasis therapy in colorectal cancer.

Keywords: Colonic neoplasm - models - animal - lymphangiogenesis

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Introduction

Colorectal adenocarcinoma is the most common malignant tumor via the lymphatic metastasis in human. Lymphatic metastasis of the tumor is an important reason for uneradicative cure and mortality. Recent studies have found that lymphangiogenesis is a major factor and mechanism for colorectal cancer lymphatic metastasis and recurrence (Achen and Stacker, 2006; Sundar and Ganesan, 2007; Nagahashi et al., 2010). With the development of research focused on identification of lymphatic vessels, technological progress to define lymphatic endothelial cell specific markers and molecular mechanism of lymphangiogenesis, it has been taken attach of the relationship between molecular mechanism of lymphangiogenesis and lymphatic metastasis, as well as the therapy based on antilymphangiogenesis (McColl et al., 2005; Wissmann and Detmar, 2006). In order to study lymphatic metastasis in colorectal cancer and antilymphangiogenic treatment, it is imperative to establish a line of lymphangiogenic animal models to research human colorectal cancer (Fidler, 2003).

Many previous studies take use of xenograft human colorectal carcinoma in nude mice as a common animal model. However, such a model of subcutaneous tumors are usually covered by fiber, leading to less lymphatic and hematogenous metastasis, so it is not exactly the performance of biological characteristics in colorectal-lymphatic or hematogenous metastasis. In recent years, scholars have reported using an orthotopic model of human colorectal cancer transplantation, in order to improve the human colorectal cancer regional lymph node metastasis rate, but no tumor lymphangiogenesis in animal models have been reported. Our experiment built an orthotopic model of human colorectal cancer transplantation ,on the basis of colorectal cancer subcutaneous xenograft model in nude mice, to explore the possibility of the establishment of human colon adenocarcinoma lymphangiogenesis model in nude mouse, in order to provide experimental models for study of human colorectal cancer lymphatic metastasis mechanisms and treatment.

Materials and Methods

Tumor cell lines

HT-29 human colon adenocarcinoma cell line:

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share in the Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology (SIBCB, CAS). Experimental animals: BALB/c-nu/nu nude mice: animal license number: SCXK (Shanghai) 2007-0005; 48 females, 6 to 8 weeks, weight 8 to 20 g, SPF (specific pathogen free in condition) level, room temperature (25 ± 1) °C, relative humidity 40% ~ 50% (Hayes Lake, experimental animals limited company).

Materials

Primary antibody for immunohistochemical (Anti-LYVE-1, Anti-D2-40, Santa Cruz, USA), biotinylated goat anti-mouse IgG secondary antibody, streptomycin affinity biotin complex (SABC) kit and diaminobenzidine (DAB) color kits, Boster (China). Western blotting blot with a primary antibody Anti- LYVE-1, Anti-D2-40, Anti-VEGF-C, Anti-VEGF-D and Anti-VEGFR-3, Santa Cruz, β-actin, Abcam, secondary antibody, Cellsignaling. RT-PCR, Trizol reagent box, Invitrogen, quantitative PCR kit, Tiangen.

Animal model of human colon adenocarcinoma orthotopic tumors

HT-29 single-cell suspension preparation: HT-29 cells cultured in 5% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml, streptomycin McCoy's 5A medium (Gibco, USA). 37.0 °C, 5% of CO_2 incubator (Forma series II of the HEPA Class 100, Thermo Company, USA). 0.25% Tyrosine, 0.02% EDTA phosphate buffer, digested, passaged, and then prepared into single cell suspension containing 1 × 10^7 cells in 0.5 ml culture medium during the logarithmic phase.

Orthotopic implantation of tumor cells to nude mouse subcutaneously: 0.2 ml of human colon adenocarcinoma HT-29 single-cell suspension (1 × 10^7/ml) were injected into the side of nude mice subcutaneously (partial dorsal) to format solid tumors using a sterile syringe. Nude mice were sacrificed by cervical dislocation after 4 weeks when the tumor diameter of 1.5 cm, tumor tissue stripped from subcutaneously tissue were placed in saline (10^5 U/L penicillin, 10^5 U/L streptavidinfactors)which containing penicillin and streptomycin, and then removed the fibrous capsule, selected a tissue block with good conditioning,which was a small piece of fish-like tumor about 1 mm in diameter, and then transplanted to the next generation of nude mice (2/generation), to be passaged to 4, the subcutaneously transplanted tumor (n = 8) was used as the source of orthotopic transplantation tumor.

Orthotopic transplantation of colon tumor to nude mouse model: 36 nude mice, fasting for 12 h before surgery were divided into two groups. One group underwent colon orthotopic implantation (n = 24); the other group injection of saline in the colon in situ as a negative control (n = 12). Colon orthotopic transplantation method: Anaesthesied with 0.5% pentobarbital sodium (45 mg/kg) by intraperitoneal injection, take a ventral midline incision , cut the abdominal wall, free cecum at the end of the colon , make inward push at the prick of the intestinal wall, to form a local intestinal concave niche; subcutaneous tumor mass of about 1.0 mm in diameter was inserted into the concave niche, using fibrin glue to cover the tumor surface the surface of and paved to the cecal wall; put back cecum into the abdominal cavity and then closed the abdomen with disinfection incision. Keep warm of the nude mice, subcutaneous injection of saline to supply water postoperatively fast water for 6 h after surgery, and provided with the high-energy diet to supplement the energy. The nude mice were observed in sub-cage to conscious-sterile and the feeding at laminar flow chamber.

Identification of lymphatic vessels generation of colon implantation of nude mice

The gross observation and HE staining: Nude body condition and activities were observed daily after inoculation, also including stool and abdominal signs; palpation the abdominal cavity in situ in order to observation of the colon tumor growth. The negative control group, orthotopic colon tumor nude mice were sacrificed by cervical dislocation, stripping the tumor tissue after 6 weeks. Tumor tissues were used for paraffin-embedded and then serial sections (thickness 4 μm), and for HE staining and immunohistochemical staining for optical microscope examination; another part of the fresh samples were cryopreserved, and used for Western blot and RT-PCR.

Detection lymphatic endothelial markers

SABC immunohistochemical staining: Slices of paraffin-embedded tumor tissue (n = 8) after treatment, dropping primary antibody anti- LYVE-1 (1:60), anti-D2-40 (1:50), biotinylated secondary antibody and SABC step by step, use DAB color box for coloring, and then conventional staining, dehydration, transparent, the sequestration to be tested. Lymph node a positive control and PBS was used as a negative control instead of primary antibody,. The judgment of results: LYVE-1 and D2-40 positive staining showed lymphatic tubular structures of the lymphatic endothelial cell membrane and cytoplasm with brown-yellow-brown particles. Periphery colon adenocarcinoma, the center and the corresponding normal tissue of the cutting edge was observed under low magnification (× 100) firstly, select one of the high lymphatic vessel density areas, and then under high magnification (× 400) counts of LYVE-1, D2-40 staining positive number of lymphatic vessels (each brown-stained endothelial cells or endothelial cell cluster, as long as the separated and neighboring vasculature, tumor cells or other connective tissue, is considered one of lymphatic vessels), that was lymphatic vessel density (LMVD). It was taken every 1 nude mouse lymphatic vessels in five high-power field as a mean of the colon adenocarcinoma center, the surrounding and normal colorectal rectal tissue LMVD.

Western blot blotting: Fresh tumor tissue (n = 8) about of 2 mm in diameter and tumor-free colon tissue (negative control, n = 4) in nude mice, extraction of protein; Configuration BCA working solution by BCA Protein Assay Kit (Shen neng, Shanghai), determination of the A562, and calculate the protein concentration; Preparation of separating gel, the plot layer of glue in the slot, and then adding the electrophoresis buffer, sample,

(Bio-Rad protein3, Bio-Rad, USA). Until the proteins were transferred to PVDF membrane (Millpro), closed membrane by adding primary antibody (Anti-LYVE-1, Anti-D2-40, Anti-β-actin; 1:1000) and HRP-labeled secondary antibody (1:1000); Adding 1:1 AB developer combined with the two anti-HRP (Tiangen, Bio-Rad, USA) imaging analysis of gray values to calculate the ratio of gray values.

Fluorescent quantitative RT-PCR: Take fresh tumor tissue (n = 8) and nude mice colon tissue (negative control, n = 4) block, extraction of total RNA by RT-PCR for mRNA detection. The primers were designed and synthesized by Invitrogen Corporation. LYVE-1: upstream 5' TGC AGA ATT ATG GGG ATC AC 3', downstream 5' GGC TGT TTC AAC TTG GTC CT 3'; D2-40: upstream 5' GGT GCC GAA GAT GAT GTG3', downstream 5' CGA TGC GAA TGC CTG TTA 3', housekeeping gene of GAPDH: upstream 5' GCA CCA CCA ACT GCT TA 3', downstream 5' AGT AGA GGC AGG GAT GAT3'. GAPDH was used as an internal control, the annealing temperature of 56 °C for 40 cycles (94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, 72 °C for 10 min). We analyzed with the ABI Prism 7300 SDS. The relative mRNA expression levels was calculated by the formula (relative mRNA expression = $2^{-\triangle\triangle Ct}$).

Detection of VEGF -C, VEGF-D , VEGFR-3

Western blot blotting: As described previously. Primary antibody, Anti-VEGF-C, Anti-VEGF-D, Anti-VEGFR-3, Anti-β-actin were diluted in 1:1000. Primary antibody and HRP-labeled secondary antibody (1:1000) by adding 1:1 AB developer combined with the two anti-HRP (Tiangen), and were imaged on the chemical luminescence imager (Bio-Rad, USA). We analysed gray value by calculation the ratio of gray value.

Fluorescent quantitative RT-PCR detection: Take 8 tissue block in 2 mm diameter in each group, extracted total RNA, tested with fluorescent quantitative RT-PCR. VEGF-C, upsream 5' GCC ACG GCT TATG CAA GCA AAG AT 3', downsream 5' AGT TGA GGT TGG CCT GTT CTC TGT 3', VEGF-D, upstream 5' CGA TGT GGT GGC TGT TGC AAT GAA 3', downstream 5' GCT GTT GGC AAG CAC TTA CAA CCT 3', VEGFR-3, upstream: 5' GAC AGC TAC AAG TAC GAG CAT CTG 3', downstream: 5' CGT TCT TGC AGT CGA GCA GAA 3', GAPDH, upstream 5' GCA CCACCAACT GCT TA 3', downstream 5' AGT AGA GGC AGG GAT GAT 3'. We analyzed with the ABI Prism 7300 SDS. The relative mRNA expression levels was calculated by the formula (relative mRNA expression = $2^{-\triangle\triangle Ct}$).

Statistical Analysis

All data analysis was by the SPSS 13.0. The experimental data was described with ± s and comparing difference between two groups using single-factor analysis of variance, $P<0.05$ was considered significant difference.

Results

Orthotopic tumor growth of the nude mice

After the colon orthotopic implantation of subcutaneously transplanted tumor about two weeks , hard nodules could be palpated in the right lower quadrant, and significantly increased at 4 to 5 weeks, up to 10 to 15 mm at 6 weeks. Some animals with weight loss, lack of exercise and cachexia. Tumor could be found at intestinal wall, pale, round or oval, with the surface of nodular protrusions, and the infiltration of surrounding tissue; profile was fish-like, larger tumor with central necrosis (Figure 1). Average tumor weight (0.76 ± 0.24) g. The rate of Orthotopic implantation was 100%.

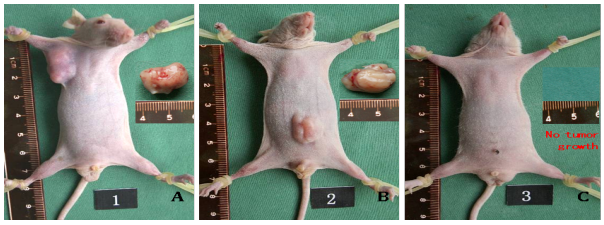

Figure 1. The Nude Mice with Xenografts and the Xenografts of Human Colonic Adenocarcinoma Cell Line HT-29 in Nude Mice. A: subcutaneous xenograft, which was used to establish in-situ xenograft. B: in-situ colonic xenograft. C: control mouse without in-situ colonic xenograft

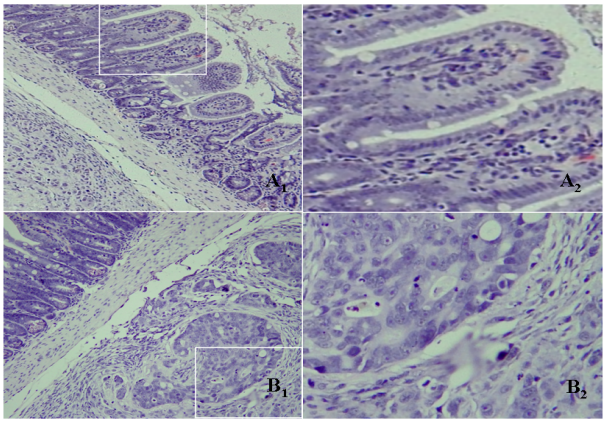

Figure 2. Histomorphologic Structures of the In-situ Colonic Xenografts and the Control Colon Without In-situ Xenograft in Nude Mice with HE Staining under Optic Microscope. A: Normal mucosa, glandular and villous in colon with a great deal of inflammatory, serous cells was observed in control group, but no infiltration of tumor cells (A1: magnification×100, A2: magnification×400). B: it was observed in the in-situ colonic xenografts that colonic wall structure was destroyed, round or oval tumor cells showed infiltrative growth, or arranged in clusters funicular, with abundant cytoplasm, deep dyeing nucleus, increased mitotic phase, connective tissue among tumor cells and part of vacuolar degeneration (B1: magnification×100, B2: magnification×400)

Control group injected with saline in the nude mouse colon, there was no tumor growth, and it is hereinafter referred to as tumor-free group.

HE staining of orthotopic implantation tumor in nude mice

Tumor free group of normal intestinal mucosa, glands, normal villi, with a large number of inflammatory serous cells, no tumor cell infiltration (Figure 2A1, 2); Structure of colon orthotopic implantation tumor was destroyed, round or oval, actively growing cancer cells showed invasive growth of cancerous tissue, arranged in a group cords, abundant cytoplasm, large nuclei deeply stained mitotic increase in cancer cells between the connective

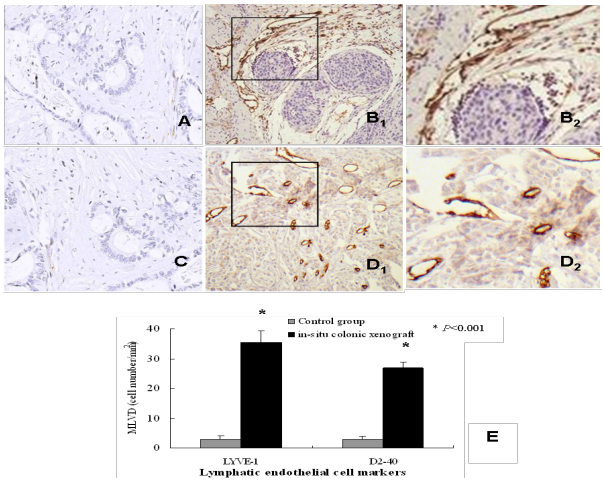

Figure 3. Immunohistochemical Staining of LYVE-1 and D2-40 (SABC method), and Number of Lymphangiogenetic Microvessels (LMVD) in the In-situ Colonic Xenografts and the Control Colon Without Xenograft in Nude Mice. A: LYVE-1 expression was negative in control group (magnification×100). B: Yellow-brown strong positive expression of LYVE-1 in the in-situ colonic xenografts was observed, and these positive tubular structures accorded with thin wall, large lumen, irregular morphological features of lymphatic capillaries, and distribution in tumor tissue and normal tissue junction. There were visible large floccules, lymphocyte, tumor cells and their fragments in the other lumen (B1: magnification×100, B2: magnification×400). C: D2-40 expression was negative in control group (magnification×100). D: Yellow-brown strong positive expression of D2-40 in the in-situ colonic xenografts, according with morphological features of lymphatic capillaries, was observed (D1: magnification×100, D2: magnification×400). E: LMVD number of LYVE-1 or D2-40 in the in-situ colonic xenografts was more than that of control group (both *P<0.001).

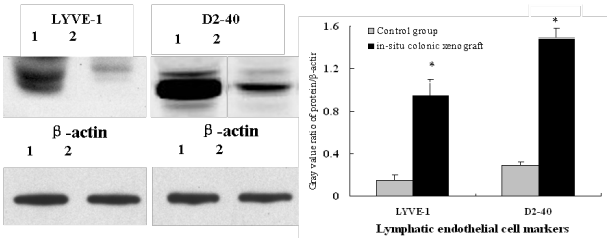

Figure 4. Western Blot Analysis of LYVE-1 and D2-40 in the In-situ Colonic Xenografts (1) and the control colon without xenograft (2) in nude mice. The expression of LYVE-1 and D2-40 proteins was significantly higher in in-situ xenografts than that of the control group (both *P<0.01)

tissue, some areas of vacuolar degeneration and necrosis of cancer cells, the structure was unclear, and could be seen a large number of inflammatory cells in the necrotic tissue.

Orthotopic implantation colon tumor of nude mouse lymphatic endothelial markers: detection of LYVE-1, D2-40

Immunohistochemical staining and LMVD: There was no or little brown tan in tumor free group of lymphatic endothelial cell markers LYVE-1 (Figure 3A), andD2-40 positive expression (Figure 3C), LMVD was very low; Colon orthotopic implantation tumor ,of which LYVE-1 (Figure 3B1, 2), and D2-40 (Figure 3D1, 2) expression

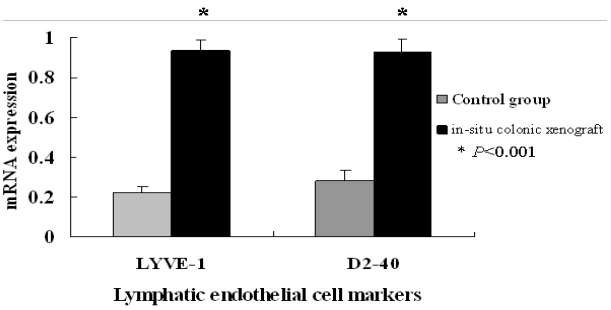

Figure 5. FFluorescence Quantitative RT-PCR Analysis of LYVE-1 and D2-40 in the In-situ Colonic Xenografts and the Control Colon Without Xenograft in Nude Mice. The expression of LYVE-1 mRNA or D2-40 mRNA was significantly higher in in-situ xenografts than that of the control group (both *P<0.001)

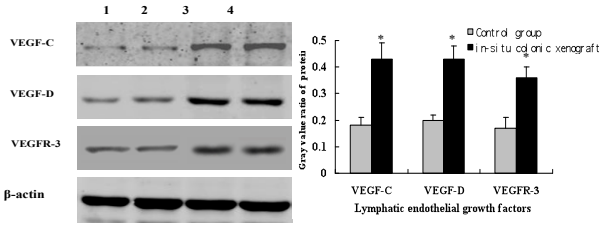

Figure 6. The Expression of VEGF-C, VEGF-D and VEGFR-3 Proteins in the In-situ Colonic Xenografts (1, 2) and the control colon without xenograft (3, 4) in nude mice (Western blot). The expression of VEGF-C, VEGF-D and VEGFR-3 proteins was significantly higher in in-situ xenografts than that of the control group (all *P<0.002)

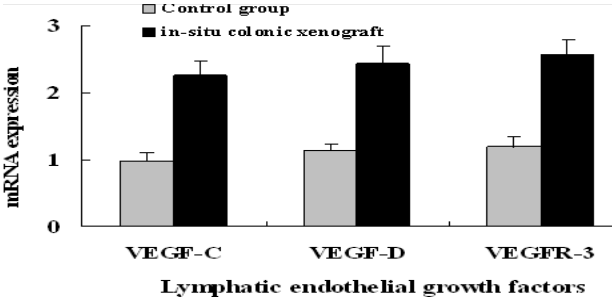

Figure 7. The Expression of VEGF-C, VEGF-D or VEGFR-3 mRNA in the In-situ Colonic Xenografts and the Control Colon Without Xenograft in Nude Mice (Fluorescence Quantitative RT-PCR). The expression of VEGF-C, VEGF-D or VEGFR-3 mRNA was significantly higher in in-situ xenografts than that of the control group (all *P<0.01)

was strongly positive. Those of LYVE-1, D2-40 positive pipeline are single endothelial cells, thin wall, large lumen, irregular, often collapse like, in line with the morphological features of lymphatic capillaries, LMVD was significantly higher than non-tumor group (P <0.001, Figure 3E), and which were distributed at the junction of the tumor tissue and normal tissue, almost no lymphatic vessels within the tumor; a large number of flocculent material, lymphocytes, tumor cells and their debris were still visible in the lymphatic lumen.

Western blot blotting: The colon orthotopic implantation tumor of nude mice, regardless of LYVE-1, D2-40 grayscale value or LYVE-1/β-actin, D2-40/β-actin, gray-scale values were significantly higher than non-tumor colon tissues, there were significant statistically differences (P < 0.001). The lymphatic endothelial

markers, LYVE-1, D2-40 protein expression also showed that the orthotopic implantation colon tumor of nude mouse coexistence of tumor lymphangiogenesis (Figure 4).

Fluorescent quantitative RT-PCR: Colon orthotopic tumors in nude mice, LYVE-1 mRNA, D2-40 mRNA expression was significantly higher than the tumor-free colon tissue ($P < 0.001$), which was in line with Western blot detection (Figure 5).

Orthotopic implantation of nude mouse colon tumor: detection of lymphangiogenic factors of VEGF -C, VEGF-D and VEGFR-3

Western blot blotting: For orthotopic implantation nude mouse colon tumors, gray value of VEGF-C, VEGF-D and VEGFR-3 were significantly higher than tumor-free colon tissue, there were significant statistical differences ($P < 0.001$) (Figure 6).

Fluorescent quantitative RT-PCR: Orthotopic implantation nude mouse colon tumor, VEGF-C mRNA, VEGF-D mRNA and VEGFR-3 mRNA in expression ,were significantly higher than tumor-free colon tissues, there were significant statistical differences ($P < 0.001$), which is in line with western blot results (Figure 7).

Discussion

In recent years, the lymphatic endothelial cell specific markers have been successively found and the molecular mechanism of lymphangiogenesis have been revealed step by step, thus the role of lymphangiogenesis in tumor metastasis, recurrence, treatment is gradually being recognized, the related research is also increasingly in-depth (Kowanetz and Ferrara, 2006; Mylona et al., 2007; Zwaans and Bielenberg, 2007; Liersch et al., 2010). Because the tumor metastasis animal models is important in cancer metastasis research, as well as lymphatic (node) metastasis as the main path of the cancer spreading take more and more attach of colorectal cancer metastasis, it is inevitably put forward higher requirements of animal models in the related mechanisms research of colorectal cancer metastasis, especially lymphatic metastasis (Tsutsumi et al., 2001; Flatmark et al., 2004; Sasaki et al., 2008). But in the past, with using of fresh cancer tissues or cell lines established in nude mice subcutaneous tumor model can not be the occurrence of deep invasion and lymph node metastasis (Fidler, 1990), while the use of nude mouse colon subserosal injection of human colorectal cancer cell lines produce orthotopic implantation of tumor, can be simulated colorectal cancer distant organ metastasis, lymphatic way to transfer is not obvious, colorectal situ lymph node micrometastasis and lymphatic generated, the development process falls far short. The reason is that tumor metastasis requires cell - cell and cell-matrix interactions between the activated cell secretory protein degradation enzymes, degradation of the matrix membrane and interstitial matrix, activation of the mobility of tumor cells invasion and metastasis-related mechanism involved in (Flatmark et al., 2004). Therefore, we needed a tumor lymphangiogenesis in animal models, to knot the study of the mechanism of rectal cancer lymphangiogenesis and lymphatic metastasis, drug intervention and anti-lymphatic therapy.

Recent 10 years, the development of tumor lymphatic metastasis is focused on the discovery of specific markers of lymphatic endothelial cells, molecular signals involved in lymphatic vessels generate, and tumor control or intervention based on these mechanisms. LYVE-1 (Jackson et al., 2001; Prevo et al., 2001; Achen et al., 2006), D2-40 (Breiteneder et al., 1999; Renyi et al., 2005; Kozłowski et al., 2011) and VEGFR-3 (Kubo et al., 2000; Achen et al., 2006; Kowanetz and Ferrara, 2006; Wissmann and Detmar, 2006) are the most important lymphatic endothelial cell-specific markers. While LYVE-1, D2-40 and VEGFR-3 expression is positive or elevated, it may confirm the lymphatic capillaries, lymphangiogenesis rather than angiogenesis. VEGF-C and VEGF-D and VEGFR-3 are recognized as lymphatic endothelial growth factor and receptors in the lymphatic-specific expression, and regulated by the VEGF-C, -D/VEGFR-3 signaling pathway (Kubo et al., 2000; Achen and Stacker, 2006; Achen et al., 2006; Kowanetz and Ferrara, 2006; Wissmann and Detmar, 2006; Sundar and Ganesan, 2007; Nagahashi et al., 2010). Our results suggest that the models of orthotopic transplantation of colorectal cancer in nude mice showed LYVE-1, D2-40 immunohistochemical staining strongly positive, LYVE-1, D2-40, VEGF-C, VEGF-D and VEGFR-3 protein and mRNA expression are significantly increased. The models confirmed the morphological features of lymphatic capillaries and the VEGF-C, -D/VEGFR-3 signaling axis control mechanism, which proved that the model of orthotopic transplantation of colorectal cancer in nude mice was a colorectal adenocarcinoma lymphangiogenesis animal model.

This experiment was successfully established an orthotopic transplantation and lymphatic metastasis model of human colorectal cancer in nude mice, it showed tumorigenesis in a short time, the high rate of tumor formation and more stable tumor pathology and biological characteristics. At the same time, observing the process of lymphangiogenesis and early lymphatic metastasis, the model simulated the natural growth of human colorectal adenocarcinoma and local infiltration process, not only in the signaling pathway but also in the morphology of lymphangiogenesis, objectively reflecting the characteristics of cancer patients' lymphatic metastasis in vivo. It provides an ideal animal model for in-depth study of lymphangiogenesis in colorectal cancer, lymphatic metastasis and antimetastatic therapy.

Acknowledgements

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