

RESEARCH ARTICLE

Establishment of and Comparison between Orthotopic Xenograft and Subcutaneous Xenograft Models of Gallbladder Carcinoma

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Abstract

Background: Gallbladder carcinoma (GBC) is the most common carcinoma of the biliary system. Among its research models, orthotopic xenograft models, important research tools, have been rarely reported in the literature however. **Aim:** To explore establishment of an orthotopic xenograft model and to evaluate the advantage and disadvantage as compared with other models. **Materials and Methods:** Subcutaneous xenograft and orthotopic xenograft models of gallbladder carcinoma in nude mice were established and compared with human gallbladder carcinomas. **Results:** For the orthotopic xenograft model and clinical gallbladder carcinomas, the lymph node metastatic rates were 69.2% and 53.3% ($p>0.05$); ascites generation rates, 38.5% and 11.7% ($p<0.05$); liver invasive rates, 100% and 61.7% ($p<0.05$); and lymphatic vessel densities (LVD), 10.4 ± 3.02 and 8.77 ± 2.92 ($p>0.05$), respectively. In the subcutaneous xenograft model, no evidence of ascites generation, lymph node metastasis and liver metastasis were found, and its LVD was lower (4.56 ± 1.53 , $p<0.05$). **Conclusions:** Compared with the subcutaneous xenograft model, the orthotopic xenograft model better simulates clinical gallbladder carcinoma in terms of metastasis and invasion, which may be attributed to the difference in microenvironment and LVD.

Keywords: Gallbladder carcinoma - orthotopic xenograft - subcutaneous xenograft - lymphatic vessel density

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Introduction

Gallbladder carcinoma (GBC) is the most common carcinoma of the biliary system and is characterized by high malignance and striking invasion (Mastoraki et al., 2010). Liver and lymph node metastasis are the major metastatic pathways of gallbladder carcinoma (Ohtani et al., 1996; Kokudo et al., 2003), and lymph node metastasis often occurs at an early stage, which leads to poor prognosis of patients with gallbladder carcinoma (Izumi et al., 1993; Tsukada et al., 1997; Kondo et al., 2002). In the past decades, scientists and doctors have exerted great efforts on the study of gallbladder carcinoma, and established various animal models as a favorable platform to observe the development of gallbladder carcinoma *in vivo*, providing valuable references for clinical studies.

As highly advocated by most researchers, orthotopic xenograft models of carcinoma can provide a suitable microenvironment for tumors and enable them to develop malignant features. However, in the study of gallbladder carcinoma, subcutaneous models were mostly used and

orthotopic xenograft models were rarely established due to the smallness of the gallbladder of the nude mouse and the fragility of the liver. Our study successfully established a subcutaneous xenograft model and an orthotopic xenograft model of gallbladder carcinoma using the NOZ cell line, and compared the two animal models with human gallbladder carcinoma in terms of growth, metastasis, pathology and lymphatic vessel density (LVD).

Materials and Methods

Patients and tissue specimens

A total of 60 cases of gallbladder carcinoma were obtained from the Affiliated Union Hospital of Fujian Medical University in China (from 2008 to 2012). The patient group was composed of 27 men and 33 women with a median age of 58 years. All patients included in this study had not received any preoperative chemotherapy or radiotherapy. The tissues were collected according to the protocol approved by the Ethics Committee of the Medical Faculty of the Fujian Medical University.

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Cell culture

The human gallbladder carcinoma cell line NOZ was originally obtained from Health Science Research Resources Bank (HSRRB) in Japan. The cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, USA). The cells were incubated at 37°C in a 5% CO₂ humidified incubator.

Establishment of the subcutaneous xenograft model

Male athymic BALB/c nude mice in an age range of 4-6 weeks were obtained from Slaccas Laboratory Animal Co., Ltd (Shanghai, China). Housing for these animals was maintained in a HEPA-filtrated environment within sterilized cages. All animals were subjected to a daily 12h light/12h dark cycle. All protocols and procedures used in the study were approved by the Institutional Animal Care and Use Committee of Fujian Medical University and were in compliance with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

NOZ cells were harvested using 0.25% trypsin-EDTA (AMRESCO, USA) and a single-cell suspension of 1×10⁶ cells (0.1 ml) was injected subcutaneously into the right armpit of 15 animals. When the tumors had grown to approximately 100 mm³, a growth record was prepared. Tumor size was measured every three days with a caliper and tumor volume was calculated by the formula: (shortest diameter)² × longest diameter × 0.5. When the tumor reached approximately 1000 mm³ in size (4 weeks after inoculation), the mice were euthanized by exposure to CO₂ and primary tumors were excised and weighed.

Establishment of the orthotopic xenograft model

NOZ cells were harvested using 0.25% trypsin-EDTA and made into a single-cell suspension of 2×10⁷ cells/ml and stored on ice. All experimental procedures were performed under aseptic conditions. All mice received a preoperative fast for 4 hours and were anesthetized with chloralhydrat (250mg/kg). After the anesthesia, about 4×10⁵ cells were mixed with Matrigel (20ul of aforementioned cell suspension in DMEM with 20ul of reduced growth factor Matrigel). The gallbladder was quickly exposed via abdominal midline incision (approximately 0.8-1.0cm) and punctured by a 31G insulin syringe (BD, USA). The bile was extruded and cleaned with a swab. Then 40ul of cell suspension mixed with Matrigel was injected into the gallbladder slowly by a 29G insulin syringe (BD, USA). The syringe was withdrawn from the gallbladder when the cell suspension became white because of solidification. Finally, the gallbladder and liver lobes were replaced, and the abdominal wall was sutured. Then the mice were put on a heating pad (37°C) for pain relief. The physical condition of the mice was monitored everyday in the first week and then every three days in the following three weeks. Four weeks later, the mice were euthanized by exposure to CO₂ and primary tumors were dissected and excised (Figure 1). In the establishment of the orthotopic xenograft model, eight nude mice were used to test the feasibility of the inoculation technique in our preliminary

experiments, and a sacrifice schedule of two animals per time after 2, 4, 6 and 8 weeks was adopted.

Hematoxylin and eosin Staining

At approximately 4 weeks after inoculation, the animals were euthanized, and the primary tumors were excised. These tumors were explanted, imaged, and immediately placed in 10% neutral buffered formalin fixative for 24 hours. The tissues were then processed and embedded in paraffin. Slides were then cut for hematoxylin and eosin (H and E) staining.

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissue samples were sectioned into serial 4 μm slices and placed on microscope slides. After deparaffinization in xylene and rehydration in alcohol, tissue sections were incubated in 3% hydrogen peroxide (H₂O₂) for 20 min to block endogenous peroxidase activity. The slides were left at room temperature overnight and warmed for 30min at 37°C prior to antigen retrieval. They were next immersed for 15 minutes in citrate buffer titrated to pH 6.0 at 95°C and rinsed with 1×PBS. The membrane permeabilization was performed using 0.3% Triton-X 100 (SENHAO, Shanghai). Nonspecific antigen reactions were blocked by 15-min incubation with 5% serum from which the secondary antibody was extracted. The slides were then incubated with the primary antibody for 12-16h at 4°C, rinsed, and further incubated with secondary antibody for 60min at room temperature. After exposure to stable 3,3-diaminobenzidine (DAB, ZSGB-BIO, Beijing) for 1-2 min, they were counterstained with hematoxylin for visualization of the nucleus. For a negative control, PBS was used instead of the primary antibody. Finally, the samples were fixed via immersion in an ethanol dilution series, final immersion in xylene, and sealed with permanent mounting medium (VECTOR, USA).

Counting of lymphatic vessel

Sections were first examined at low magnification

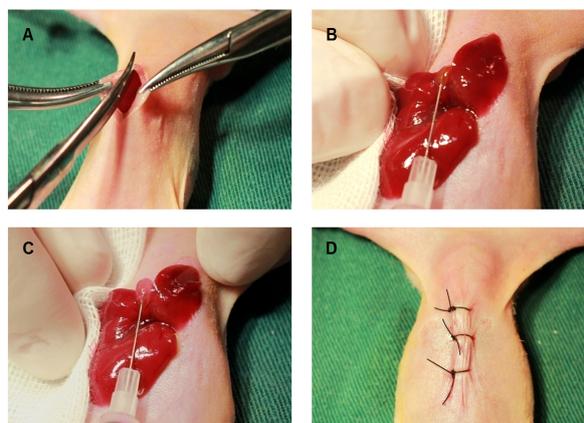


Figure 1. Surgical Procedures of the Orthotopic Xenograft Model of Gallbladder Carcinoma. A). Abdominal incision into abdominal cavity; B). Puncturing gallbladder, and extruding bile; C). Injecting single-cell suspension (mixed with Matrigel) into the gallbladder, and pulling out the syringe when the cell suspension became white; D). Suturing abdominal wall

Table 1. Ascites or Metastasis of the Xenograft Models and Human Gallbladder Carcinoma

	Ascites	Liver invasion or metastasis	Lymph node metastasis	Lung metastasis	Nevin Stage IV-V
Subcutaneous xenograft models	0/15	0/15	0/15	0/15	0/15
Orthotopic xenograft models	5/13 (38.5%)	13/13 (100%)	9/13 (69.2%)	0/13 (0)	13/13 (100%)
Human gallbladder carcinoma	7/60 (11.7%)	37/60 (61.7%)	32/60 (53.3%)	0/60 (0)	44/60 (73.3%)

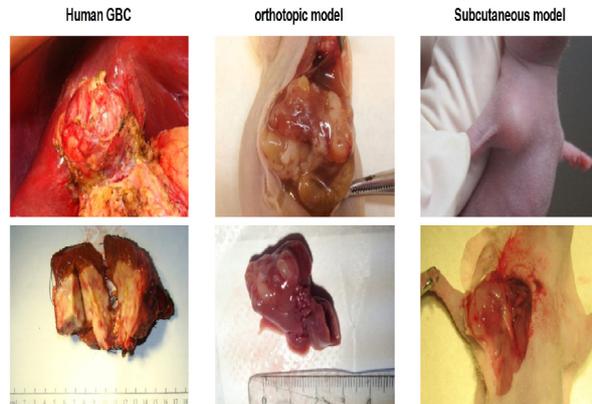


Figure 2. Tumor Growth of Human GBC and the Xenograft Model. The tumor of the orthotopic xenograft model demonstrated invasive growth with irregular shape and unclear boundary as shown in human gallbladder carcinoma. The tumor of the subcutaneous xenograft model was approximately round, and the tumor could be completely separated because of their intact capsule

($\times 100$) to identify areas with most intense staining and apparent highest density of microvessel (hotspot) (Weidner et al., 1991). Three areas of hotspots were selected by three pathologists who independently evaluated the slides for microvessel counting using $400\times$ magnification (0.17 mm^2 field), without the knowledge of patient status. In the absence of hotspots, three or more randomly selected areas were counted. Brown vessels without muscle layer or red blood cells (RBCs) in their lumen were considered as lymphatic vessels. Single immunoreactive endothelial cells, or endothelial cell clusters separate from adjacent microvessels, were counted as a vessel (Vermeulen et al., 1996). The highest number of vessels counted was recorded and used in the statistical analysis.

Statistics

The results were presented as the mean \pm SEM. Statistical analyses were done with the SPSS software 17.0 (using chi-square test for enumeration data, employing t-test for means comparison between two groups and one-way analysis of variance for means comparison among multi-groups). $p < 0.05$ was considered statistically significant.

Results

Establishment of the nude mice models

The eight orthotopic xenograft mice in the pre-experiment were planned to be sacrificed separately after 2, 4, 6 and 8 weeks. At post-inoculation week 2, the first two mice were sacrificed. The tumor size was found to be relatively small and no metastasis occurred.

At post-inoculation week 4, invasion, metastasis and a little of ascites were observed in the next two mice, whose physical status was fine. However, approximately at post-inoculation week 6, one mouse died, and the remaining three mice showed dyscrasia and were close to death. So all of them were sacrificed at the end of week 6, and significant weight loss and massive ascites were observed in them. Therefore, we decided to establish 15 cases of the orthotopic xenograft model and sacrifice them 4 weeks after inoculation. Of the 15 orthotopic xenograft mice, one died on the 3rd day after inoculation because of abdominal bleeding; one did not show any tumors in the abdominal cavity; and the rest 13 were inoculated successfully. The tumor formation rate was 86.67%. Fifteen cases of the subcutaneous xenograft model were inoculated successfully, and the tumor formation rate was 100%.

The growth of gallbladder carcinoma

The tumors of human gallbladder carcinoma showed significant invasive growth, such as irregular shape, unclear boundaries, absence of capsules and so on. Similar invasive growth was observed in the tumors of the orthotopic model. The boundaries between the tumor and adjacent tissue were unclear, so it was difficult to measure these tumors or to weigh them. Expansive growth was seen in the tumors of the subcutaneous xenograft model. The tumors were wrapped by capsules, and the boundaries of them were clear. The average volume of the tumors was $(2135.1 \pm 642.5)\text{ mm}^3$, and the average weight was $(2.398 \pm 0.621)\text{ g}$. Representative images were shown in Figure 2.

Metastasis and ascites

The metastasis and ascites of the xenograft model were analyzed and shown in Table 1. Among the 60 cases of human gallbladder carcinoma, 7 cases reported ascites; 32 cases, lymph node metastasis; 37 cases, liver invasion; and 1 case, simultaneous presence of liver metastasis and abdominal metastasis (Figure 3A). Forty-four cases of human gallbladder carcinoma progressed to Nevin stage IV-V. In all 13 orthotopic xenograft mice, liver invasion or metastasis was observed. Nine cases of lymph node metastasis and 5 cases of ascites were found (Figure 3B). No lung metastasis was detected in all 13 mice. In all subcutaneous xenograft mice, neither metastasis nor ascites appeared (Figure 3C).

There is no significant difference in the lymph node metastatic rate between orthotopic xenograft model (69.2%) and clinical gallbladder carcinoma (53.3%) ($p > 0.05$). Ascites generation rate, liver invasive rate and stage IV-V rate of orthotopic xenograft model were higher than those of clinical gallbladder carcinoma (38.5% vs

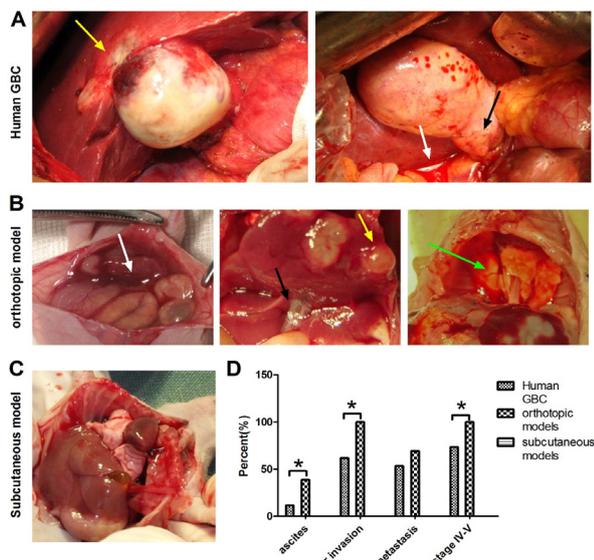


Figure 3. Ascites or Metastasis of Human GBC and the Tumors of the Xenograft Model. Orthotopic xenograft tumors, similar to human GBC, showed strong invasion or metastasis. **A and B.** Ascites, liver metastasis and lymph node metastasis were respectively indicated by white, yellow and black arrows. Green arrow pointed to the lung of mice; **C.** Liver metastasis, lymph node metastasis and lung metastasis were not observed in the subcutaneous model; **D.** Ascites generation rate, liver invasive rate and stage IV-V rate of the orthotopic xenograft model were higher than those of clinical gallbladder carcinoma (* $p < 0.05$)

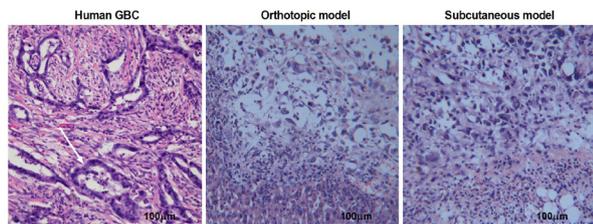


Figure 4. H-E Staining of Human GBC and the Tumors of the Xenograft Model. The two kinds of xenograft tumors and human GBC showed striking atypia, for example, large nucleus, different size, irregular shape, and so on. The tumor cells of gallbladder adenocarcinoma featured an adenoid-like arrangement (indicated by white arrow). Masses of tumor cells with moderate cytological atypia were observed in the subcutaneous model and orthotopic model. Scale bars=100um

11.7%, 100% vs 61.7% and 100% vs 73.3%, respectively) ($p < 0.05$, Figure 3D).

H-E staining

In this study, 83.3% (50/60) cases of the clinical GBC were adenocarcinoma. As shown in Figure 4, tumor cells featured an adenoid-like arrangement and significant stroma reaction was visible around the tumors. Tumor cells of the orthotopic model and subcutaneous model grew into masses, and no obvious histological difference was found between the two models.

Lymphatic vessel density (LVD)

Lymphatic vessel of xenograft tumors and human gallbladder carcinoma was respectively marked by LYVE-1 antibody and D2-40 antibody by immunohistochemical

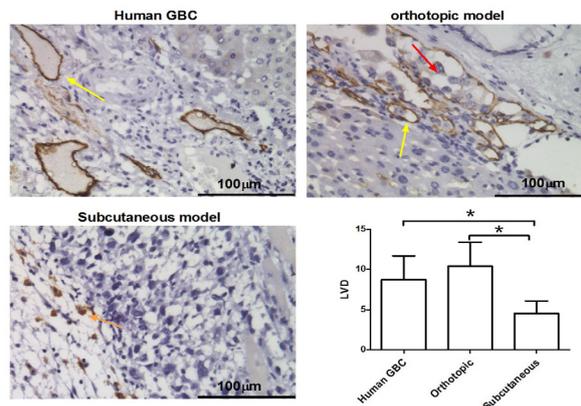


Figure 5. Lymphatic Vessel Density (LVD) of Human GBC and two Xenograft Models. Immunohistochemical was used to detect the lymphatic vessels. Typical brown positive structure was indicated by yellow arrow. Obvious brown lymphatic vessels were observed in the human GBC and orthotopic xenograft tumors, and some of the lymphatic vessels were invaded by tumor cells (red arrow). Little tubular structures were observed in subcutaneous xenograft tumors, only some brown punctate structures (orange arrow), which possessed nucleus and maybe single lymphatic endothelial cells, were seen in the field. The LVD of orthotopic xenograft tumors and human gallbladder carcinoma was significantly higher than that of subcutaneous xenograft tumors. * $p < 0.01$. Scale bars=100um

method. Brown vessels (indicated by yellow arrow in Figure 5) without muscle layer or red blood cells in their lumen were considered as lymphatic vessels. Brown single immunoreactive endothelial cells, or endothelial cell clusters (orange arrow, Figure 5), separate from other MVs, were counted as a vessel. The LVD of orthotopic xenograft tumors (10.39 ± 3.02) and human gallbladder carcinoma (8.77 ± 2.92) were significantly higher than that of subcutaneous xenograft tumors (4.56 ± 1.53) ($p < 0.01$).

Discussion

In this study, we established the orthotopic xenograft model and subcutaneous xenograft model of gallbladder carcinoma, and found the orthotopic xenograft model presented a better picture of the development of clinical gallbladder carcinoma than the subcutaneous xenograft model in terms of lymph node metastasis, ascites generation, liver metastasis and LVD.

The growth, invasion or metastasis of tumors depends mainly on the interactions between tumor cells and the microenvironment of the host organ. The grafting site of the orthotopic xenograft model is similar to the primary site of tumors in human body, which provides a microenvironment equivalent or similar to that of the human body. This microenvironment promotes the tumor invasion or metastasis (Fidler, 1990). In our study, no lymph node or distant metastasis was observed in the subcutaneous xenograft model; and meanwhile, the tumors were covered with a capsule. In contrast, in the orthotopic xenograft model, distinct hepatic and lymph node metastasis were noted, and the tumors were of high invasiveness. Such variations indicate that the organ environment can influence the invasiveness of the tumor

and its potential of metastasis. The findings of our study conform to the related reports of previous studies (Fidler 1991; Kuo et al., 1995). In the experiment we found the liver metastatic rate of the orthotopic model was even higher than that of clinical gallbladder carcinoma, which may be due to the cases that we chose. All the cases involved in the study were clinically considered suitable for surgery and were in a relatively early tumor stage. Those late or terminal patients without surgical treatment and pathological diagnosis were not enrolled in our study. In a word, the orthotopic xenograft model with a proper transplantation environment and identical metastasis path can better simulate the development of clinical patients with gallbladder carcinoma.

In recent years, researchers have made lots of efforts to establish the orthotopic xenograft model. Horiuchi et al. (2003) directly injected the NOZ cells into the gallbladder after ligating the cystic duct, which prevented tumor cells from entering the duct. But the accompanying lymphatic vessels were also ligatured, which changed the physiological environment around the gallbladder. Meanwhile, some researchers argue that, due to the treatment of tumor cells with trypsin, the original tumor tissue structures are impaired, which results in the poor differentiation tendency of the transplanted tumor, and the tumor fragment is a more favorable material for transplantation (Furukawa et al., 1993). Although Chang, et al successfully inoculated subcutaneous tumor fragment in the surface of the gallbladder, their tumor fragment came from the cell suspension that was treated with trypsin (Chang et al., 2007). The aforementioned poor differentiation tendency is not successfully checked. In 2007, Egberts et al. (2007) reported an ameliorated method of establishing the orthotopic xenograft model of gallbladder carcinoma, in which they suspended the Mz-ChA-1 cells in matrigel and injected them directly into the gallbladder. However, they didn't describe in detail the injection of tumor cells. In our pre-experiments we inoculated tumor cells by the method that they described and found it difficult to achieve satisfactory outcomes. When we "aspirated gallbladder juice", we found the gallbladder wall was easily torn. So we improved their method by gently puncturing the gallbladder wall with a thin needle (31G syringe), squeezing out the bile, and then oppressing gallbladder using a gauze for a few seconds before injecting tumor cells. The improved procedures successfully emptied the gallbladder and prevented tumor cell leakage. Egberts et al did not describe how the syringe used for inoculation of tumor cells was chosen. In our study, the first several attempts showed that the gallbladder wall was vulnerable to a thick needle and that a too thin syringe was easily blocked by the cell suspension. Finally, we chose the insulin syringe of 29G, and achieved good results.

The gallbladder cancer cell line NOZ screened and used in this study was isolated from the metastatic ascites of a 48-year-old female patient of gallbladder cancer, which is of high invasiveness and strong metastatic potential and suitable for transplantation into nude mouse; the morphology of transplanted tumor maintains the features of its primary cells (Homma et al., 1988).

Through the comparison of subcutaneous transplantation between our study using NOZ cells (NOZ group) and other domestic studies using GBC-SD cells (GBC-SD group), we found the tumor size of our NOZ group at the 4th week was larger than that of their GBC-SD group (Zhao et al., 2005). Given the longer doubling time and slightly smaller size of NOZ cells *in vitro*, in comparison with those of GBC-SD cells, This result indicates that the biological features of tumor cell *in vivo* and *in vitro* might not always conform to each other, or the *in vivo* environment of nude mice is probably more agreeable to NOZ cells than to GBC-SD cells, which indirectly illustrates the importance of establishing animal models.

Researchers have proved that lymphatic metastasis is a major pathway for the spread of gallbladder carcinoma, in which lymphangiogenesis is a critical link (Skobe et al., 2001, Saharinen et al., 2004). Meanwhile, some clinical research reported that lymphangiogenesis could function as an important evaluation index in the prognosis of tumor (Detmar and Hirakawa 2002, Chang et al., 2011, Muniz et al., 2011). In our study, we detected lots of lymphatic vessels in both clinical gallbladder carcinoma and orthotopic xenograft tumors and found high invasive and metastatic rate in both cases. On the contrary, neither lymph node metastasis nor liver metastasis could be observed in the subcutaneous xenograft model, which showed low lymphatic vessel density. Taken together, our findings are in line with the previous reports and prove that LVD may be an important factor for the metastasis of gallbladder cancer. We believe that the lymphatic vessels can serve as a pivotal candidate for the inhibition of tumor metastasis. Future investigation into the molecular mechanism of lymphangiogenesis and the targeted anti-tumor therapy are strongly recommended and will become fruitful research hotspots.

Acknowledgements

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