



## Experimental study on anti-angiogenesis of recombinant mouse angiostatin gene in mice with gallbladder carcinoma

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### ABSTRACT

Gallbladder cancer is one of the gastrointestinal tumors with an extremely poor prognosis. Its incidence rate is gradually increasing worldwide, and the rate of radical resection surgery is extremely low. Not sensitive to radiotherapy and chemotherapy, with a very poor prognosis. This study aimed to investigate whether the recombinant mouse angiostatin gene transfected anti-angiogenic gallbladder cancer cells can express angiostatin protein with the activity of inhibiting the growth of vascular endothelial cells and the inhibitory effect on the growth of gallbladder cancer. The recombinant mouse angiostatin gene eukaryotic expression plasmid was transfected into the gallbladder cancer cell line by applying liposome LIPOFECTAMINE 2000, and its activity was detected by vascular endothelial cell proliferation analysis. The results show that angiostatin can inhibit the growth of transplanted gallbladder cancer, and as the number of injections increases, the inhibition rate of gallbladder cancer growth also increases. At the end of the experiment, the total inhibition rate of gallbladder cancer growth reached 95% 5%, 20%, 30%, 40% gradually increase. Therefore, angiostatin has potential clinical application value in gene therapy of gallbladder cancer.

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### Introduction

The growth of tumors depends on the abundant blood supply. By inhibiting tumor neovascularization, tumor ischemic necrosis can be achieved for therapeutic purposes. The growth and metastasis of tumors are closely related to angiogenesis. Gallbladder cancer is one of the gastrointestinal tumors with an extremely poor prognosis, and its incidence rate is gradually increasing worldwide. The rate of radical resection surgery is extremely low, and it is not sensitive to radiotherapy and chemotherapy, so it is of great significance to actively explore gene therapy for gallbladder cancer. At present, there are positive reports on the role of angiostatin in inhibiting tumors such as fibrosarcoma, melanoma, gastric cancer and prostate cancer. Gallbladder cancer is a highly malignant tumor, and its effect on angiostatin has not been reported.

Gallbladder cancer has a very poor prognosis, so it is of great significance to actively explore gene therapy for gallbladder cancer. STROM compared 84 newly diagnosed patients with histologically

confirmed gallbladder cancer with 126 controls without stones and 264 controls without cholelithiasis or common bile duct stones. In contrast, the race is a very high-risk factor for gallbladder cancer in BOLIVIA (1). Subbannayya used MIF inhibitors, ISO-1 and 4-IPP or their specific siRNAs to perform in vitro cell assays in a group of gallbladder cancer cell lines. In invasive cell lines, 654 proteins were found to be overexpressed, while 387 proteins were down-regulated (2). Tian's study found that early diagnosis, careful perioperative evaluation and precise surgery are necessary factors to achieve good results in IGBC treatment (3). Ma analyzed ten retrospective studies involving 3191 patients. The study found that in patients with LN-positive disease and AJCC stage that reached or exceeded A1CC, the greatest benefit of AT was also observed over stage II tumors, but not in patients with LN-negative or R0 disease (4). The Lian study found that JUNB levels in GBC samples were elevated and were positively correlated with PDK1 levels in tumors. Therefore, PDK1 acts as a

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tumor promoter in human GBC by up-regulating JUNB (5).

Angiostatin is a degradation product of plasma plasminogen and fibrinolytic enzyme. There are many studies on angiostatin. Tykhomyrov's research found that angiostatin is an effective angiogenesis inhibitor and anti-inflammatory molecule, which plays a vital role in the pathophysiology of non-cancerous breast diseases (6). The expression and significance of Liang angiostatin, vascular endothelial growth factor and matrix metalloproteinase-9 in cerebral ischemia-reperfusion was measured in diabetic rats (7). Guzyk's research found that PARP-1 inhibition therapy can prevent the formation of PARP-1 and GFAP cleavage derivatives. In the retina of anti-PARP-treated diabetic animals, partial reversion of angiostatin levels was shown (8). Cao studied biodegradable polymer vesicles co-delivery of angiostatin and curcumin for anti-angiogenic therapy (9). İsmail studied the level and clinical significance of angiostatin in diabetic patients receiving insulin therapy and determined the serum angiostatin level by ELISA (10). Gerlicz-Kowalczyk assessed the level of angiostatin in the serum of SSC patients. The results showed that compared with healthy controls, the AS levels of SSC patients were significantly different, and suggested that impaired angiogenesis may lead to low AS levels (11). It can be seen that there are many ways to produce angiostatin in vivo, and different pathways may produce different angiostatin-like plasminogen degradation fragments.

In this article, gallbladder cancer cells transfected with recombinant mouse angiostatin gene express angiostatin protein with inhibitory effect on the growth of vascular endothelial cells and the inhibitory effect on the growth of implanted gallbladder cancer in nude mice. The recombinant eukaryotic expression plasmid pcDNA3.1 (+)-angiostatin of recombinant mouse angiostatin gene was transfected into gallbladder cancer cell line GBC-SD by applying lipofectamine2000, and C418 resistance screening was performed. The expression of angiostatin was detected by western blot. Nude mice were inoculated and the tumor volume and tumor microvessel density were compared. The results show that angiostatin can inhibit the growth of transplanted gallbladder cancer, and as the number of injections increases, the inhibition rate of gallbladder cancer growth also

increases. 5%, 20%, 30%, 40% gradually increase. The innovation of this study is to introduce this eukaryotic expression vector into human gallbladder cancer GBC.SD cells, culture it with a selective medium containing a selection concentration of neomycin G418, randomly select resistant clones and expand the culture. The growth curve of tumor cells in vitro before and after transfection was measured; immunofluorescence cytochemistry and western blot were used to detect the expression of angiostatin protein in GBC.SD cells transfected with positive plasmid. The inhibitory effect of angiostatin secreted by GBC.SD cells transfected with positive plasmid on the proliferation of vascular endothelial cells were detected. This laid a foundation for further research on the significance of angiostatin in gallbladder cancer ant vascular gene therapy and the feasibility of a clinical application.

## Materials and methods

### Experimental materials

The E. coli DH5a strain and the eukaryotic expression vector pcDNA3.1 containing the CMV promoter are kept by the virus room of a provincial people's hospital. Angiostatin (angiostatin) cDNA gene fragment and rabbit anti-HATAG polyclonal antibody were kindly donated by Xining Hospital of a military medical university. Horseradish peroxidase-labeled goat anti-rabbit secondary antibody, Western blot kit: Bo ± De. Gene sequencing: Shanghai Shantou Biotechnology Company. Fetal bovine serum and calf serum: Hangzhou Sailing Company. Recombinant human basic fibroblast growth factor (BFGF): In-nitrogen. Lipofection AMINE 2000, G418 and DMEM medium: Gibco. λDSDNA/HindI standards and various restriction endonucleases: HUAMEI Company or NEWENGL AND Biolabs. Plasmid extraction and gel recovery kit: Shanghai Hua shun Company. Gallbladder cancer cell line GBC-SD: Shanghai Institute of Cell Biology, Chinese Academy of Sciences, cultured in 15% (volume fraction) calf serum, high glucose DMEM culture medium, 37C, 5% (volume fraction) CO<sub>2</sub> Vascular endothelial cell line ECV-304: a stemmatological hospital of a military medical university, cultured in a DMEM culture medium containing 10% (volume fraction) fetal bovine serum and high glucose at 37 C, 5% (volume fraction) CO<sub>2</sub>. The specific composition ratio

of the culture medium is shown below (Table 1), which involves LB liquid medium and LB solid medium.

**Table 1.** Specific composition table of bacterial culture medium

LB liquid medium		LB solid medium	
Composition per L medium		Composition per L medium	
Tryptone	10g	Tryptone	15g
Yeast extract	5g	Yeast extract	5g
NaCl	10g	NaCl	10g
--	--	Agarose	15g

### Construction and grouping of mice angiostatin eukaryotic expression vector

PEDNA3.1(+)-angiostatin plasmid construction and gene transfection. After digestion, recovery and ligation, the angiostatin DNA gene fragment was loaded into the eukaryotic expression vector PEDNA3.1(+), transformed, selected resistant clones and purified the plasmid was confirmed by enzyme digestion and gene sequencing (12). Continuous culture of gallbladder cancer cell line GBC-SD, lipofectamine2000 gene transfection, set experimental group (angiostatin group) pCDNA3.1 (+)-angiostatin 1.2 $\mu$ g / liposome 3 $\mu$ L, negative control group (empty vector control group) PEDNA3.1 (+) 1.2  $\mu$ g / liposome 3  $\mu$ L, blank control group liposome 3  $\mu$ L. After 48 hours of transfection, the cells in the blank control group died after being cultured with the selective medium containing the selected concentration of neomycin C418 (400 mg / L) for 1wk. The experimental group and the negative control group showed visible cell clones after 4wk. Random selection of resistant clones, after the expansion of the cells, were transferred to the culture flask with a 200 mg / L G418 culture medium to expand the culture. Another wild GBC-SD cell as a control. At the same time, the *in vitro* growth curve of the three groups of tumor cells was determined: cultured with 150mL / L calf serum without G418 and high-glucose DMEM culture medium, and cells in logarithmic growth phase  $1 \times 10^*$  were inoculated into a 24-well culture plate.

The extraction and purification of plasmid pCDNA3.1 (+) were as follows:

(1) Add 250  $\mu$ l of P1 solution to the bacterial pellet and shake until completely suspended. (2) Add 250 $\mu$ l of P2 solution and immediately invert the centrifuge tube gently 5 to 10 times to mix. Let stand at room

temperature for 4 minutes. When extracting multiple samples at the same time, add one tube, mix one tube, and start timing when the first tube is added (13). (3) Add 350 $\mu$ l of P3 solution and immediately invert the centrifuge tube gently 5 to 10 times to mix. When extracting multiple samples at the same time, add one tube, mix one tube, and the timing starts when the first tube is added. (4) Centrifuge at 12000g ~ 13000g for 10 minutes. The supernatant was carefully transferred to the adsorption column and centrifuged for 15 seconds. Discard the liquid in the collection tube and place the adsorption column in the same collection tube. (5) Add 500  $\mu$ l of B1 solution to the adsorption column, centrifuge for 15 seconds, pour off the liquid in the collection tube, and place the adsorption column in the same collection tube. (6) Add 500 $\mu$ l of W1 solution to the adsorption column, centrifuge for 15 seconds, pour off the liquid in the collection tube and place the adsorption column in the same collection tube. (7) Add 500 $\mu$ l of W1 solution to the adsorption column, let stand for 1 minute, and centrifuge for 15 seconds. Discard the liquid in the collection tube and place the adsorption column in the same collection tube. (8) Centrifuge for 1 minute. (9) Put the adsorption column in a clean 1.5ml centrifuge tube, add 50 $\mu$ l TI solution in the center of the adsorption membrane, let stand at room temperature for 1 minute, then centrifuge for 1 minute, store 1.5ml centrifuge tube (plasmid DNA solution) at -20 $^{\circ}$ C (14).

### Data measurement and processing

Eleven Blab / e nude mice (age 4-6 wk.) were randomly divided into 2 groups, and a  $1 \times 10^*$  / L logarithmic growth cell suspension 0.2 mL was injected subcutaneously in the back of the nude mice. Six in the angiostatin group and five in the wild GBC-SD cell control group were injected with GBC-SD/PEDNA3.1(+)-angiostatin cells and GBC-SD cells. After 30 days of observation, the nude mice were sacrificed, the tumors were excised and their long diameter and short diameter b (unit mm) were measured, and the tumor volume was calculated according to the formula  $1/2 \times a \times b^2$ .

The above two groups of tumor specimens were prepared into paraffin sections, and immunohistochemical staining was performed according to the SABC method, anti-rabbit anti-mouse VWF MAB, working concentration 1: 200,

secondary antibody 1: 200 diluted horseradish peroxidase label Sheep anti-rabbit IgG, after DAB coloration, vascular endothelial cells were stained brown-yellow (15). Evaluate the staining results, randomly count the number of blood vessels in 10 200-time fields, and take the average value as the MVD value of the nude mouse tumor, calculate the average MWD value of the same group of nude mouse tumors. Statistical processing: use SSPS 12.0 for data processing. P <0.05 is considered statistically significant.

**Results and discussion**

**Measurement results of tumor cells after angiostatin gene introduction**

The tumor volume of the angiostatin gene introduction group was significantly different from that of the blank control group (Table 2). Before the introduction of the angiostatin gene, tumor growth was observed. There was no significant difference in tumor volume between the blank control group and the angiostatin gene introduced group. After the introduction of angiostatin in the angiostatin gene introduction group, the tumor growth became slow, while the tumor in the blank control group grew rapidly, significantly faster than the angiostatin gene introduction group.

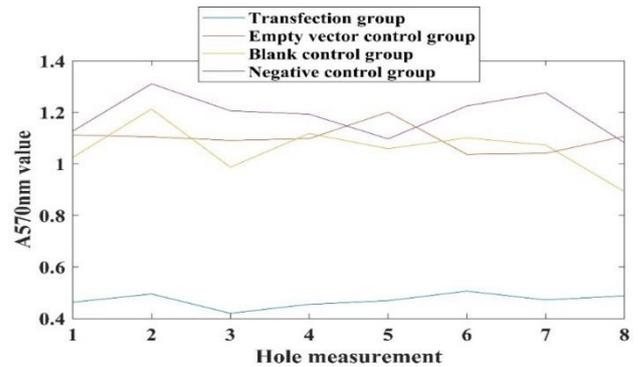
**Table 2.** The volume of gallbladder cancer tumors in recombinant mice

Grouping	Time after planting				
	1W	2W	3W	4W	5W
Blank control group	0.46±0.22	6.44± 3.03	23.64±5.42	69.10±18.88	91.02±11.09
Angiostatin gene introduction group	0.44±0.21	1.71±0.45	4.41±1.82	4.58±2.46	4.74±2.38

**Recombinant mouse angiostatin gene transfection anti-angiogenesis analysis on cell proliferation of gallbladder carcinoma mice**

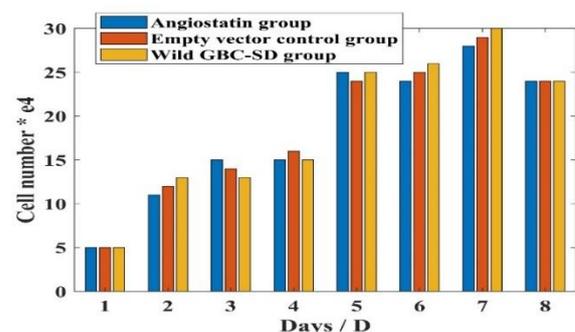
The MTT test was performed with ECV-304 cells (Figure 1). The As70m value of the transfection group was 0.471 ± 0.026, the A57m value of the empty vector control group was 1.099 +0.051, and the As70mm value of the blank control group was 1.058 ± 0.087. The As70mm value of the group was 1.189 ± 0.073. P <0.05 between the transfection group and the negative control group, and P> 0.05 between the

empty vector control group and the blank control group and the negative control group. It is proved that the supernatant secreted by the transfection group can effectively inhibit the proliferation of vascular endothelial cells stimulated by BFGF, and this effect is specific to endothelial cells. The supernatant of GBC-SD cells transfected with blank vector and the supernatant of wild GBC-SD cells had no such effect.



**Figure 1.** A570nm value of each hole in the MTT test

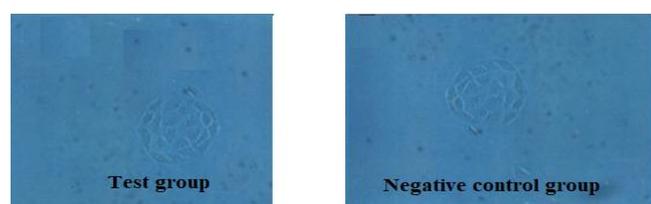
After screening with 400 mg / L G418 for 1 week, all cells in the blank control group died, and some cells in the experimental group and the empty vector control group survived. After 4 weeks, cell clones appeared in both groups. The single clones were expanded and observed under a light microscope. There was no significant difference in cell morphology between the experimental group and the empty vector control group. The number of cell growth was shown (Figure 2). Angiostatin group was no significant difference in cell growth rate and doubling time between the control group and wild GBC-SD group, indicating that peDNA3.1 (+)-angiostatin gene transfection does not directly inhibit the growth and proliferation of gallbladder cancer cells in vitro.



**Figure 2.** Cell growth status of each group

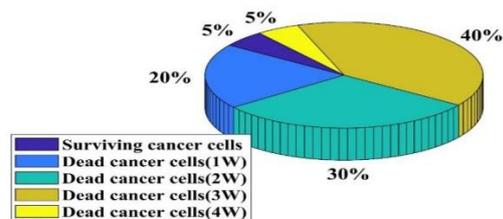
### Expression of angiostatin gene in gallbladder cancer cells and analysis of treatment results

In order to effectively express the mouse angiostatin gene in eukaryotic cells, we used liposome Lipofect AMINE 2000 to transfect the recombinant eukaryotic expression plasmid pcDNA3.1 (+)-angiostatin into human gallbladder cancer cell GBC-SD. After screening with G418 at a concentration of 400ug / ml for 1 week, all cells in the blank control group died, and some cells in the experimental and negative control groups survived. After 4 weeks, cell clones appeared in both groups (Figure 3). The single clones were expanded and observed under a light microscope. There was no significant difference in cell morphology between the experimental group and the negative control group.



**Figure 3.** Cell cloning in G418 screening

In this experiment, angiostatin, which inhibits the production of vascular endothelial cells, is directly injected into the transplanted gallbladder cancer through gene transfer to study whether anti-tumor angiogenesis therapy is effective for breast cancer and the status of anti-angiogenesis. It was found that angiostatin can inhibit the growth of transplanted gallbladder cancer, and as the number of injections increases, the inhibition rate of gallbladder cancer growth also increases. At the end of the experiment, the total inhibition rate of gallbladder cancer growth reached 95% (Figure 4). Gradually increasing from 5%, 20%, 30%, 40%. In addition, the survival time of mice in the angiostatin gene introduction group was 56 days, while that in the blank control group was 36 days. The treatment of angiostatin significantly prolonged the survival time of the mice. The microvessel density ( $96.8 \pm 69.2 / \text{mm}^2$ ) of tumor tissue in the angiostatin gene introduction group was significantly lower than that in the blank control group ( $475 \pm 163.2 / \text{mm}^2$ ), indicating that the gene introduction of angiostatin can be inhibited by inhibiting the formation of tumor neovascularization the growth of the tumor.



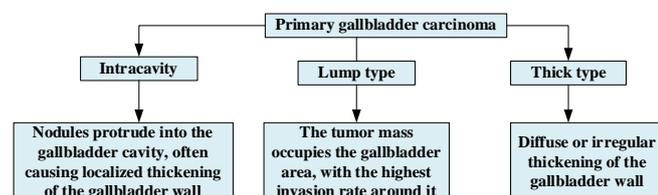
**Figure 4.** Inhibition rate of gallbladder cancer growth

The cause of gallbladder cancer is still unclear, and susceptibility may be related to repair gene polymorphisms. At present, the high-risk factors for gallbladder cancer that are uniformly recognized at home and abroad are as follows: cholelithiasis, gallbladder polyps, abnormal bile pancreatic duct fusion, porcelain-like gallbladder, diabetes, gender (females, especially prolific women), segmental gallbladder adenomyosis, obesity, smoking, internal and external estrogen, chronic inflammatory bowel disease abdominal oblique is a high-risk factor for gallbladder cancer (16). The risk of developing gallbladder cancer is twice as high as those without diarrhea, colon polyps, syndrome, family history of gallbladder cancer, postoperative gallbladder fistula, eating habits, age (increased incidence of people older than the age), surgical treatment Gastrointestinal diseases are related to gallbladder cancer, occupational factors (in the chemical industry, oil refining, shoemaking, textile, paper making, etc., the incidence rate in rural areas is higher than in cities), bile infections (biliary tract infections of Salmonella and mixed bacteria, Helicobacter pylori) (17).

Studies now suggest that bacterial infections of the biliary tract may cause tumorigenesis and development through the following mechanisms: (1) Induce the host to produce carcinogens. (2) Inhibit the immune response of the host body, causing rapid tumor development. (3) A few bacteria can produce estrogen-like substances, thereby promoting tumor growth (18). (4) Some bacteria become the host of oncogenic viruses, and viruses and bacteria work together to promote the occurrence of tumors (19). (5) In the occurrence and development of gallbladder cancer, it may be the result of the combined action of aerobic bacteria and anaerobic bacteria. Chronic cholecystitis can often cause gallbladder mucosa organization; calcification of part of the gallbladder mucosa is also a high-risk factor for gallbladder cancer (20). Among the many high-risk factors for

gallbladder cancer, the most common clinically are gallstones and gallbladder polyps (21).

Imaging examination methods for gallbladder cancer include ultrasound, endoscopic ultrasound, CT, MRI, MRCP, fluorine 18 labeled deoxyglucose positron emission tomography, endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography, Angiography, etc. Imaging of primary gallbladder cancer is often divided into three types (Figure 5): intracavity type: nodules protruding into the gallbladder cavity, often causing localized thickening of the gallbladder wall. Mass type: cancer mass occupies the gallbladder area, with the highest invasion rate around it. Thick-walled type: diffuse or localized irregular thickening of the gallbladder wall (22).



**Figure 5.** Imaging types of primary gallbladder cancer

Gallbladder cancer is extremely malignant, difficult to diagnose early, and has poor therapeutic effects. These all bring great challenges to clinical treatment. The average survival time of gallbladder cancer patients is months, and the annual survival rate is less than (23). Part of the reason for such a high mortality rate is due to abnormal anatomical features: there is no membrane layer between the gallbladder wall and the adjacent liver, the surrounding connective tissue is connected to the liver connective tissue, and tumor cells are more likely to invade and metastasize to the liver. Surgery is still an effective method for the treatment of gallbladder cancer, and the necessity and therapeutic effect of radiotherapy, chemotherapy and other adjuvant treatment methods need further research (24). In general, the existing treatment methods have not greatly improved the survival rate and prognosis of patients. Therefore, exploring new treatments for this devastating disease is a hot topic in current research (25).

Neovascularization is necessary for tumorigenesis, growth and metastasis, and inhibition of this process can inhibit tumor growth (26). Angiostatin can strongly inhibit the growth of vascular endothelial

cells and angiogenesis (24, 27). Among the 38,000 proteins, it is the enzymatic hydrolysis product of plasminogen (23). It contains the first 4 KRINGLE regions of plasminogen but complete fibrinolysis Proenzyme does not show angiogenesis inhibitory activity (22, 28). Treating tumors by inhibiting angiogenesis has become another strategy to overcome tumors. This therapy is called dormancy therapy, which inhibits the growth and metastasis of malignant tumors by blocking angiogenesis for a long time (19). Animal experiments have confirmed that this method can inhibit tumors such as fibrosarcoma, melanoma, gastric cancer and liver cancer, and has the advantages of no tolerance, no toxicity, a broad tumor suppressor spectrum and accessible target cells. If the transgenic method can be used to transfer the angiostatin gene into the target cells to make it autocrine and paracrine angiostatin. Not only can the cost be reduced, but also a high concentration of drugs can be caused locally in the tumor to enhance the therapeutic effect (17, 26).

Due to the instability of cancer cell genes, the diversity of cancer cell types and the high mutation rate of genes, the above-mentioned anti-cancer drugs have little effect on improving the survival rate of patients with solid tumors, especially those with metastatic tumors. The toxic and side effects of anti-cancer drugs on normal cells and the resistance of tumor cells to these drugs have long troubled people. The target cell for anti-vascular endothelial cell therapy is endothelial cells, which do not have a high degree of instability of tumor cells, so it is not easy to produce drug resistance. Tumor endothelial cells divide very quickly, 50 times that of normal endothelial cells, which makes them an excellent therapeutic target for angiogenesis inhibitors.

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#### Interest conflict

None.

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