

Experimental study on the prevention of liver cancer angiogenesis via miR-126

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Abstract. – OBJECTIVE: The aim of the present work was to study the prevention of liver cancer angiogenesis via miR-126. For this purpose, experimentations were conducted.

MATERIALS AND METHODS: The precursor sequence of miR-126 was amplified in the DNA of human liver cancer cell lines. We, therefore, constructed the overexpression and interference vectors of miR-126 *in vitro*; which were respectively transferred to liver cancer cells in the logarithmic phase and inoculated under both sides of the back skin of Balb/c-nu nude mice aged 4-6 weeks with 10⁶ (1 x 10⁶) cell suspension. The experiment consisted of non-vector control group, miR-126 overexpression group, and miR-126 inhibition group. Eight weeks later, the mice were sacrificed; the tumor volumes and serum ALT, AFP, VEGF levels were compared. VEGF expression, as well as the microvascular density of the liver tissues, was detected via immunohistochemistry.

RESULTS: Tumors volumes, serum ALT, AFP and VEGF levels and positive rates of VEGF were low in the miR-126 overexpression group and high in the miR-126 inhibition group, the difference being statistically significant ($p < 0.05$).

CONCLUSIONS: At the end of this study, we conclude that miR-126 inhibits liver cancer angiogenesis.

Key Words

miR-126, Liver cancer, Angiogenesis, VEGF, Microvascular density.

Introduction

It is well known and properly demonstrated that tumor is composed of tumor cells and tumor blood vessels¹. Tumor cells promote the development of angiogenesis while angiogenesis, in turn, promotes the development of tumor and provides necessary paths for tumor metastasis. Liver cancer is a malignant tumor with high blood supply, high blood transfer and high recurrence rate, and angiogenesis may play an important role in its malignant biological behavior². Studies on miRNAs molecules thou-

ght these molecules were closely associated with tumor cell proliferation, differentiation, apoptosis and other biological behaviors³. Abnormal expression of miRNAs in liver cancer includes miR-126, -21, -31, -16, etc., in which miR-126 may promote or inhibit cancer in a variety of tumors⁴. The relation between miR-126 and angiogenesis in liver cancer has not received unified agreement. Therefore, by building a model of liver cancer, this study analyzes the possible mechanism to inhibit liver cancer angiogenesis via miR-126 intervention.

Materials and Methods

Material Sources

Human liver cancer cells HepG2 were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) cell culture fluid consisting of 10% fetal calf serum (FCS), 1000 U/ml penicillin, 100 mg/ml streptomycin purchased from Gibco BRL (Grand Island, NY, USA). Cells were cultured at 37°C in 5% CO₂, 95% O₂.

Vector Construction and Cell Transfection

TRIzol and transfection reagent lipidosome (LipofectaminTM2000) were purchased from Invitrogen (Carlsbad, CA, USA). MicroRNA RT Kit and RT-PCR kit were purchased from Applied Biosystems (Foster City, CA, USA); DNA extraction kit and SYBG Green RT-PCR kit were purchased from TaKaRa (Dalian, China); 24-well plate, 96-well plate and cell culture dish were purchased from Costar (Carlsbad, CA, USA); pCDN3.1 and pCDNA-Sponge-Ready empty vector were purchased from R&D (Hercules, CA, USA); and primer sequence synthesis and sequencing were completed by Shenzhen Huada Gene Research Institute (China).

MiR-126 precursor sequence in the DNA of HepG2 genome was expanded, the upstream primer being expanded as 5'-CTCGAGTCGTAC-CGTGAGTAATAAT-3' and the downstream primer being expanded as 5'-AAGCTTTCGTAC-CGTGACATAATG-3'; conditions were expanded as 5 min of 94°C initial denaturation, 30 s of 94°C denaturation, 30 s of 54°C anneal, 30 s of 72°C extension, 30 cycles and 10 min of 72°C. Amplification products used Xho I and Hind III as insertion sites; fragments of miR-126 prosomatifera were inserted into pCDNA3.1 vector via genetic recombination and sequencing was identified to construct miR-126 overexpression vector. Two oligonucleotides (TCGTACCGTGAGTAATAATGCG) with the length of 45 bp from repetitive miR-126 reactive sequences were synthesized and inserted into pCDNA-Sponge-Ready empty vector via annealing and sequencing to build the interference vector for miR-126.

Liver cancer cells in the logarithmic phase were collected. They were washed with PBS twice, digested by pancreatin and, then, counted as 5×10^5 cells, were inoculated in the 6-well plate for 24h of cultivation. The cell experimental groups consisted of empty vector control group, miR-126 overexpression group, and miR-126 inhibition group. According to the liposome transfection instructions, 5 μ l of transfection reagent Lipofectmine2000 was mixed with 2 μ l plasmid, put under indoor temperature for 20 min, added slowly into culture supernatant fluid, and further cultivated after being shaken to blending. Twenty-four hours later, fluorescence was observed, and each group of cells went down to the future generation with a proportion of 1:10.

Construction of Heterograft Transplantation Tumor Cancer Cell Model

Cells in each group in the logarithmic phase were digested into single-cell suspension. Living cells were observed as over 95% via trypan blue staining method, and the density was adjusted so that the density of tumor cells was 1×10^6 cells/ml. One hundred microliters (1×10^5) of cell suspension was inoculated under both sides of the back skin of nude mice (Shanghai Silaike Experimental Animal, Co. Ltd.), which were killed after normal raising for 8 weeks and the tumor bodies were taken for test.

Observation Indexes and Test Methods

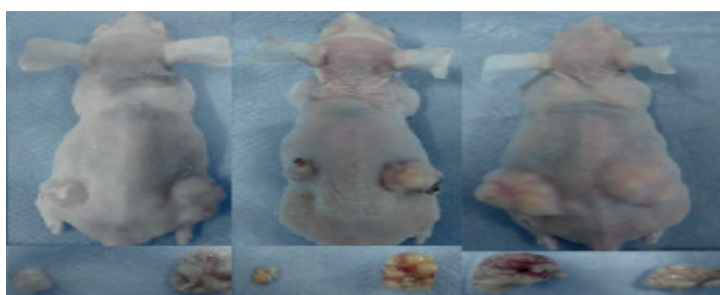
Serum ALT, AFP, and VEGF levels were compared, and VEGF expression of cancer liver tissues,

as well as microvascular density, were examined via the immunohistochemical method. Five ml of venous blood from the rat tail was collected and sent for examination 20 min after centrifugation for 20 min. ALT and AFP levels were detected by the automatic biochemical detector, and the VEGF levels were detected by ELISA. Reagent kits were purchased from Zhongshan Biological Technology Co., Ltd. (Beijing, China), and carried out in accordance with the instructions. Mouse-anti-human VEGF, CD34 monoclonal antibody, biotin labeling goat-anti-mouse IgG, horseradish peroxidase labeling streptavidin were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Immunohistochemistry included the following main steps: conventionally make paraffin embedding tissue section, dewax to water, antigen repair, block endogenous peroxidase activity, block antigen, add mouse-anti-human VEGF or CD34 monoclonal antibody working liquid (1:100) drop by drop, put in a wet box at 4°C for 18 h, and incubate at 37°C for 1 h; wash with phosphate-buffered saline (PBS) for 2 min x thrice, add biotin blockers (1:10) drop by drop, incubate in a wet box at 4°C for 20 min, wash with PBS for 2 min x thrice, add biotin goat-anti-mouse IgG working fluid (1:200) drop by drop, incubate in a wet box at 37°C for 30 min, wash with PBS for 2 min x thrice, add horseradish peroxidase labeling streptavidin working fluid (1:200) drop by drop, incubate in a wet box at 37°C for 30 min in the box, color with DAB, redye with hematoxylin, dehydrate to transparent, preserve with neutral gum sealing piece, and observe under the microscope. Results were interpreted in the following way: replace primary antibodies with PBS as negative control, and take normal liver tissue slice staining as positive control; take dead endothelial cells of liver cancer cells as positive cells; select 5 high vision (200 x) randomly in each area, with the area containing most endothelial cells dyed brown as a "heat island"; record the number of positive cells, and calculate the average microvascular density (MVD).

Statistical Analysis

SPSS 19.0 (SPSS Inc., IBM SPSS Statistics for Windows, Armonk, NY, USA) was used for entering and analysis. Quantitative data were represented as average \pm standard deviation, and intergroup comparison was conducted via single factor ANOVA analysis followed by Post Hoc test (LSD). Qualitative data were represented by a number of cases or percentage (%), and intergroup comparison was examined by the χ^2 -test: $p < 0.05$ refers to statistically significant difference.

Figure 1. Comparison of tumor volumes (from left to right are the control group, overexpression group and inhibition group respectively).



Results

Comparison of tumor volumes

Tumor volumes were smallest in the miR-126 overexpression group, and largest in the miR-126 inhibition group, the difference being statistically significant [control group (425.3 ± 36.2), miR-126 overexpression group (316.7 ± 45.3), miR-126 inhibition group (586.4 ± 56.9) mm^3 , $F=24.326$, $p < 0.001$] (Figure 1).

Comparison of Serum ALT, AFP and VEGF Levels

Comparison of serum ALT, AFP and VEGF levels in each group before the experiment reflected no statistically significant difference ($p > 0.05$); serum ALT, AFP and VEGF levels in each group raised after the experiment, with the lowest levels in the miR-126 overexpression group and the highest in the miR-126 group. The difference was statistically significant ($p < 0.05$) (Table I).

Results of Immunohistochemistry

Positive rates of VEGF and CD34 were lowest in the miR-126 overexpression group and highest

in the miR-126 inhibition group, the difference being statistically significant ($p < 0.05$) (Table II and Figure 2).

Discussion

MiR-126 is positioned at human chromosome 9q34.3 at the gene sequence part of coded endothelial cell growth factor-like receptor 7. It plays a role in the occurrence and development of different types of tumors and its expression drops in non-small cell lung cancer, breast cancer, brain cancer and colon cancer to inhibit cancer suppressor gene⁵. Studies mirror that miR-126 can target at vascular endothelial growth factor (VEGF), adjust the occurrence and neogenesis of blood vessels, and participate in the occurrence and transfer of liver cancer, breast cancer, lung cancer and oral cancer⁶.

This research found that: tumor volumes, serum ALT, AFP and VEGF levels, and positive rates of VEGF and CD34 were low in the miR-126 overexpression group and high in the miR-126 inhibition group, the difference being statistical-

Table I. Comparison of serum ALT, AFP, and VEGF levels.

Group	ALT (U/L)		AFP (U/ml)		VEGF (ng/L)	
	Pre-experiment	Post-experiment	Pre-experiment	Post-experiment	Pre-experiment	Post-experiment
Control group	23.2±6.4	75.9±10.2	0.6±0.1	7.7±2.5	123.5±33.2	223.4±52.6
miR-126 overexpression group	21.4±6.2	51.2±11.4	0.5±0.1	5.2±2.1	130.4±35.5	176.5±57.8
miR-126 inhibition group	22.6±6.5	94.8±15.3	0.5±0.1	11.3±3.3	128.2±34.6	316.3±54.9
<i>F</i>	0.634	9.627	0.968	8.456	0.825	12.321
<i>p</i>	0.725	<0.001	0.754	<0.001	0.634	<0.001

Table II. Results of Immunohistochemistry (%).

Group	VEGF	CD34
Control Group	15.6±3.3	17.2±4.1
miR-126 overexpression group	10.3±3.2	11.4±4.2
miR-126 inhibition group	21.2±3.7	25.3±4.3
<i>F</i>	9.625	10.632
<i>p</i>	<0.001	<0.001

ly significant. This indicated that miR-126 could inhibit cancer genes in liver cancer, inhibit VEGF activity and vascular endothelial cell neogenesis, and work as an important mechanism to inhibit liver cancer development⁷. The study thought that tumor blood vessel neogenesis was related to vascular endothelial cell proliferation, excitation of angiogenesis like VEGF, and the expression of matrix metalloproteinases (MMPs)⁸. ALT, AEP and VEGF levels in the miR-126 overexpression group obviously dropped, indicating that miR-126 could improve liver function while inhibiting tumor development.

Angiogenic factors mainly include vascular endothelial cells, tumor cells, protein growth factors and steroid secreted from stroma cells, which share the common characteristic to start and promote angiogenesis. VEGF is the most important promoting factor for angiogenesis that has been detected and identified till now, which combines with tyrosine kinase receptor to exert different

biological functions⁹. Many studies have found that many miRNAs can regulate angiogenesis. For instance, miR-210 and -130a can promote angiogenesis, while miR-126, -221 and -222 can inhibit angiogenesis^{10,11}. Now, how miRNAs molecules promote or inhibit tumor angiogenesis mechanism remains unclear, but specific molecules on the surface of tumor angiogenesis may become potential target spots¹². Among the five members of VEGF family, VEGF-A plays the most important role in angiogenesis, as it can combine VEGFR-2 and VEGFR-1. The combination of VEGF-A and VEGFR-2 can promote endothelial cell proliferation and angiogenesis; while the combination of VEGF-A and VEGFR-1 can show diverse functions on angiogenesis as local microenvironment changes¹³. Angiogenic inhibitors with VEGF, VEGFR and its signal path as targets achieve gratifying achievements *in vitro* cell experiments, animal models and some clinical researches, safe and effective to improve the prognosis of tumor patients^{14,15}.

Conclusions

To sum up, miR-126 can inhibit the angiogenesis of liver cancer, and its specific inhibiting mechanism can be further explored to provide more theoretical evidences for the clinical application of miR-126

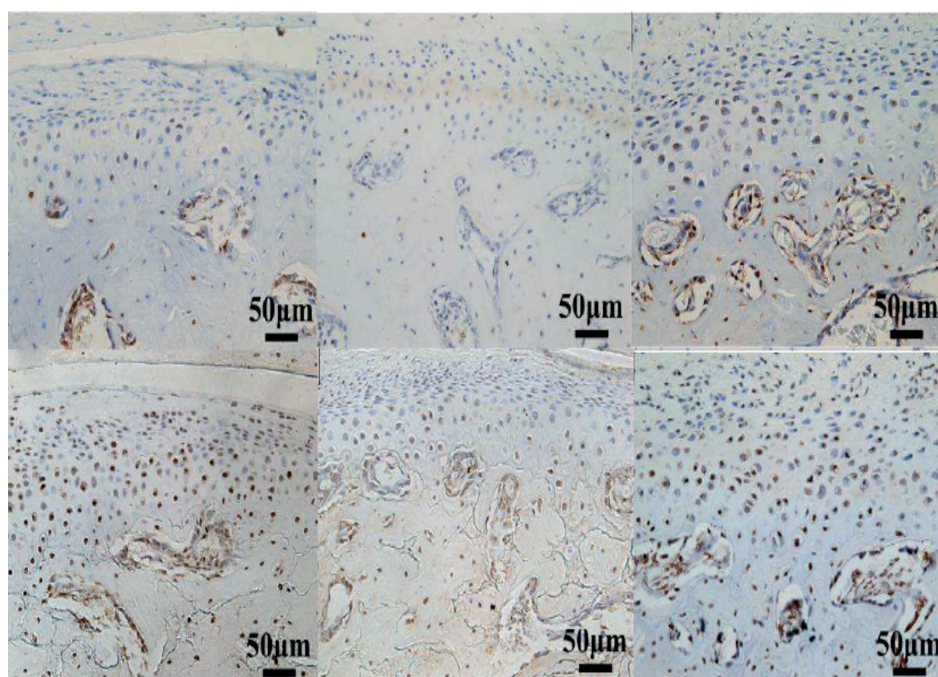


Figure 2. Immunohistochemical staining (200×, the row above is VEGF, the row below is CD34; from left to right are control group, overexpression group and inhibition group respectively).

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Conflict of Interest:

The Authors declare that they have no conflict of interests.

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