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Mir-135a inhibits tumor cell proliferation by regulating the expression of notch pathway in hepatoblastoma

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ABSTRACT

MiRNA affects the proliferation, migration and cycle of tumor cells. However, the mechanism of regulating Notch pathway expression and inhibiting tumor cell proliferation in hepatoblastoma is not clear, we need to further explore. In this study, the dact2 gene can inhibit liver fibrosis. In this experiment, we used in vitro culture of hepatoblastoma cells and flow cytometry to detect the effect of miRNA-135a on the tumor cell cycle. The expression of miRNA-135a was detected by real-time PCR in 6 tumor samples and normal controls to observe and analyze the expression level of the important signal pathway proteins in the cells after mir-135a transfection. The cells were divided into the experimental group and the control group. The experimental group was transfected with miRNA-135a cells, while the control group was not transfected. Finally, the data are analyzed and discussed. from the third day, the activity of M17 cells in the mir-135a group began to be inhibited. By the fifth day, the inhibition rate of M17 was 50.64% ($P < 0.01$), and M21 was 25.02% ($P < 0.01$). In the experimental group, 53.38% of the cells were in the G1 phase, and mir-135a could block the tumor cells in G1 phase, affect the cell cycle process, and then affect cell proliferation.

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Introduction

The regulatory role of miRNA may be of great significance for the study of gene function, human disease prevention and control, and biological evolution. The process of miRNA synthesis in animals and plants is different (1-3). In animals, miRNA is first transcribed into primary primirna (4-6) by RNA polymerase II. Liver fibrosis and cirrhosis caused by chronic hepatitis virus infection are the most important risk factors for liver cancer (7-8). The low survival rate and high recurrence rate of HCC patients are largely due to the formation of hyperactive angiogenesis and microvascular infiltration (9-10).

More and more people pay attention to the role of miRNA in the development of HCC, especially in the study of the vascular infiltration mechanism of HCC. MiR-125b and mir-100 can directly target or indirectly down-regulate the expression of angpt2, interfere with the angiogenesis of tumor cells, and inhibit the metastasis of liver cancer (11-13). The down-regulation of mir-29b expression indicated that HCC patients had a low tumor-free survival rate, which inhibited the angiogenesis and invasion and metastasis of HCC by regulating the expression of MMP2 (14-16). Mir-125a can target to inhibit MMP11 or VEGF-A and HCC metastasis.

Mir-151 can promote intrahepatic metastasis of HCC by targeting RhoGDIa to activate NaCl, cde42, rhogtpases, etc. MiR-26a targeted to regulate hepatocyte growth factor (HGF), and then affect hgfcmet signal, angiogenesis and metastasis (17-19). Tgf-mir-34a-ccl22 axis causes accumulation of T cells regulated by portal vein system, destroys immune microenvironment, and leads to proliferation and vascular metastasis of scattered tumor cells (20-22). LncRNA can interact with DNA, RNA and protein to realize the diversification of its functions (23). In recent years, it has been found that lncRNA plays an important role in tumor development (24). LncRNA-h19 and IGF2 are a pair of imprinted genes. H19 promotes tumor development by regulating related genes such as angiopoietin and FGF18 and activates

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the miR-200 family through acetylation, thus inhibiting metastasis and epithelial-stromal transformation of hepatoma cells (25). In addition, some lncRNA, such as hulc, pandar, carlo-5, afap1-as1 and so on, are related to the vascular infiltration of HCC.

In this paper, mir-135a can inhibit the proliferation of hepatoblastoma cells by regulating the expression of the Notch pathway. Therefore, the method of control experiment is adopted. By analyzing the relevant data, mir-135a inhibited the proliferation of hepatoblastoma cells by regulating the expression of the Notch pathway, the results showed that 53.38% of the cells in the experimental group were in the G1 phase; mir-135a could block the tumor cells in the G1 phase, affect the cell cycle process, and then affect the cell proliferation. Inhibit the proliferation of tumor cells.

Materials and methods

All the hepatocytes in the experimental center were purchased from the ATCC cell bank and approved by the hospital ethics committee. Cells were divided into experimental and control groups. Mirna-135a cells were transfected in the experimental group, but not in the control group.

Cell Culture

M17 cell line complete medium: DMEM medium containing 5% fetal bovine serum (BSA). Culture conditions: 37 °C, 5% CO2. Cell resuscitation: take 5ml of complete medium, 15ml of the centrifuge tube, add a pipette gun, and incubate in a 37 °C water bath; take the frozen cells out of the liquid nitrogen tank, quickly put them into a 37 °C water bath, shake them from time to time, and make them completely melt for about 3min, pay attention to the time should not be too long, discard the complete medium in the bottle on the platform without bacteria, moisten the cells with 2ml Hank's solution, and remove dead cells and residual serum; Discard Hank's solution, centrifugate it at 2000rpm for 5min, and discard the supernatant; scatter the cells at the bottom of the heart tube with light elasticity, add 1ml of complete culture medium into the centrifugation tube, gently pat it with pasteurization tube for 3-5 times, and make single-cell suspension; add 200 suspension into 25cm2 culture bottle according to 1:5 dilution channel method, supplement the complete culture medium to 6ml, and culture in 37 °C 5% CO_2 incubator. Cell count, wipe the slide of blood cell counting plate covered with alcohol, dry it with ear ball, and test under a microscope.

MiRNA Transfection

A certain number of cells were inoculated into the pore plate (the number of inoculated cells was determined according to the area of the bottom of the pore plate and the action time of the transfection reagent), and cultured in a 37 °C 5% CO2 incubator; when the cells adhered to the wall, they were usually transfected with miRNA after 24 hours of culture (the number of cells can be adjusted); the transfection solution was prepared according to the instructions of the transfection reagent: dilute the appropriate amount of mir-135a, The basic solution of serum-free and double antibody free opti Memi transfection with the same pore volume of NC and aseptic is to add them at room temperature for 5 minutes, put them at room temperature for 15 minutes, and then add the transfection solution to the corresponding porous culture plate, gently shake and incubate them in 37 °C and 5% carbon dioxide incubator; expression of mir-135a; extraction and separation of tumor tissue, Cut the tumor tissue obtained in the hospital and normal tissue into 50-100g, and refrigerate them at - 80 °C; 2) take out the frozen tissue samples, put them into the mortar, add 2-4ml triazole according to the tissue volume, grind them into powder under liquid nitrogen, and fully split them; put the tumor tissue at room temperature for about 30min, and take the Trizol homogenate of sclera into 1.5ml EP tube after Trizol melting, 1ml each tube, and refrigerate them at - 80 °C for standby. The RNA precipitates were dried at the bottom of the tube; 20 nitroglycerin containing 1%RNase inhibitor were added to each tube to dissolve the RNA precipitate, and the 1lrn cell cycle was detected by Nanodroplet absorption.

Detection of Cell Cycle

In each period of the cell cycle (G0, G1, s, G2, m), DNA content showed periodic changes, and DNA content in the G1 phase remained diploid; after entering the S phase, DNA began to synthesize, and DNA content in the nucleus was between g1-g2 phase; when cell replication ended to quadruple, it

entered G2 phase, and existed in G2 phase in quadruple form. PI can specifically bind to DNA in cells. There is a good linear relationship between the fluorescence intensity and the binding amount of PI. Based on this principle, DNA content in cells can be measured, G1, s and G2 phases of cells can be distinguished, and the percentage of each phase can be calculated by the software.

Western Blot Method

Assemble the electrophoresis device according to the operating instructions of the electrophoresis device, add ddH2O for leakage detection; prepare the separation glue, add it into the board, seal it with water, make the separation glue level flat, 37 °C, light cure for 40 minutes; prepare the concentrated glue, insert the comb, 37 °C, light cure for 40 minutes; install the device into the electrophoresis tank, and add enough electrode buffer, the electrode liquid in the inner tank shall not be too short board, and the electrophoresis liquid in the outer tank shall not be too short board; Example loading: add 20-50 μ G protein sample to form a solidified sample adding hole, add 1 × SDS loading buffer and the same number of adjacent protein samples to eliminate the edge effect at both ends of the hole, and add appropriate protein markers; electrophoresis: adopt constant pressure mode, when the sample is concentrated, the voltage is set to 80V, the purpose is to concentrate protein. When the sample was transferred to the separation gel, the voltage was adjusted to 140V. The electrophoresis time of the sample in the separation gel can be adjusted according to the molecular weight of the tested protein. In general, electrophoresis is stopped when the lower edge gel of Bromphenol blue is stopped, then the film is transferred: after electrophoresis, the gel is removed and the target fragment is removed, and the gel is removed rapidly and immersed in the electroplating liquid. Select 2umnc film and adjust the film conveyor to 0.5cm in black. White to red principle is placed in the electric transfer tank in the ice water mixture, 300mA constant current membrane, the membrane transfer time is adjusted according to the molecular weight of the protein to be measured; sealing: take out the NC membrane after the membrane transfer, put it into the prepared 5% sealing solution, shake it slowly at room temperature for more than 2.5h; membrane cleaning: PBST washing for 10min × 3 times; combining with anti-1: dilute anti-1 with western blot anti-dilution solution, and then combine with the corresponding NC membrane, Room temperature combined for more than 2.5h (incubation at 4 °C overnight is better); membrane washing: PBST washing for 10min × 3 times; combined with secondary antibody: dilute secondary antibody with a certain amount of closed solution, combine with corresponding NC membrane, incubation at room temperature for 1.5h; membrane washing: PBST washing for 10min × 3 times; exposure: exposure with Odyssey exposure scanner.

Results and discussion

Mir-135a Expression

According to the statistical analysis of data, as shown in Figure 1 and Table 1, the expression of mir-135a in tumor cells is significantly lower. We used real-time PCR to further verify the expression level of mir-135a in normal control and 6 tumor cell samples. Mir-135a was highly expressed in the normal control group and significantly decreased in 6 tumor cell samples ($P < 0.01$), which was consistent with the core analysis of miRNA expression in tumor cells.

Table 1. Mir-135a expression

	1	2	3	4	5	6
Normal tissue	1	1.03	1.02	1	1.03	1.05
Tumor tissue	0.01	0.09	0.1	0.12	0.06	0.05

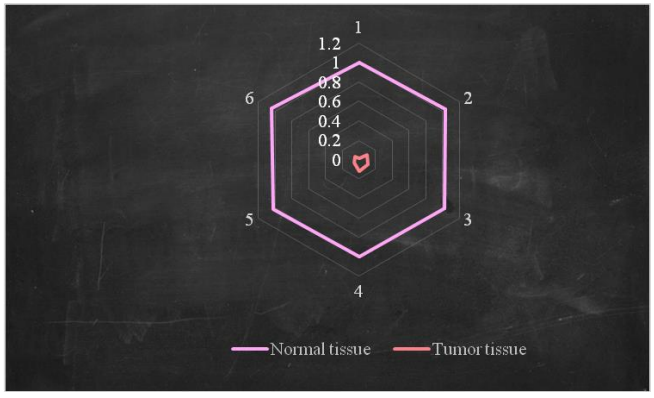

Figure 1. Mir-135a expression

Tumor Cell Effect

According to the statistical analysis of data, as shown in Figure 2 and Table 2, in order to further understand the impact of mir-135a on tumor cell proliferation, we detected the impact of mir-135a on tumor cell proliferation by flow cytometry. The effect

of mir-135a on tumor cell proliferation was observed in 5 days. The results of 5 days were drawn into the growth curve. From the 3rd day, the activity of M17 cells in the mir-135a group began to be inhibited. By the 5th day, the inhibition rate of M17 was 50.64%, and M21 was 25.02% ($P < 0.01$).

Table 1. Tumor cell effect

	1	2	3	4	5
Test group	0.4	0.6	0.8	0.9	0.7
control group	0.3	0.5	0.9	1.3	1.4

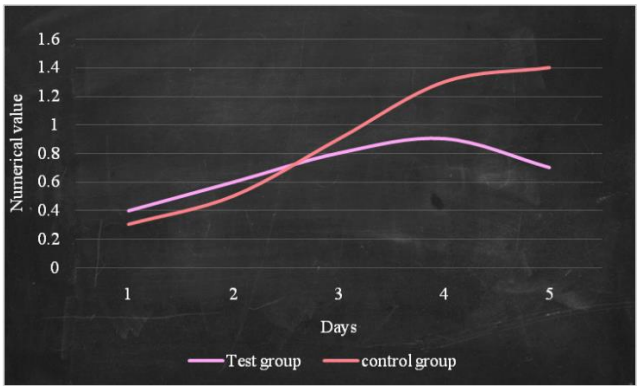

Figure2. Tumor cell effect

Cell Cycle

According to the statistical analysis of data, as shown in Figure 3 and Table 3, the G1 / s stage is an important test point. After mir-135a transfection, the G1 phase of the control group accounted for 71.86%, and the G1 phase of the test group accounted for 53.38%; mir-135a can block the G1 phase tumor cells, affect the cell cycle process, and then affect cell proliferation.

Table 2. Cell cycle

	G1	G2	S
Test group	53.38%	11.34%	35.28%
control group	71.86%	9.01%	19.13%

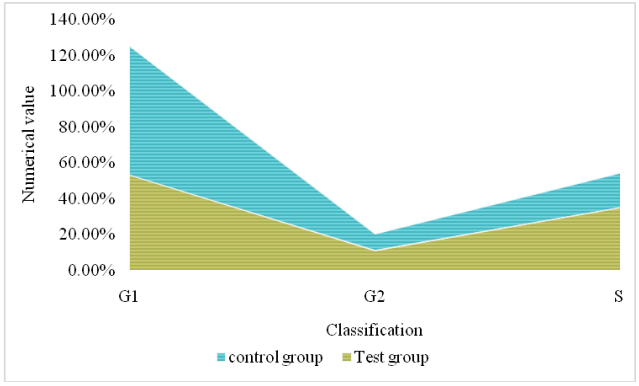

Figure 3. Cell cycle

Protein Expression

According to the statistical analysis of data, as shown in Figure 4, M17 tumor cell lines were divided into the control group and mir-135a group. Compared with the control group, SIRT1 protein expression in the mir-135a group was significantly lower.

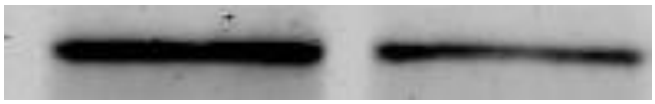

Figure 4. Protein expression. Compared with the control group (right), SIRT1 protein expression in the mir-135a group (left) was significantly lower.

MicroRNA is closely related to tumor formation. The mechanism of tumorigenesis involves many aspects (26). The change of these genes will lead to the out-of-control of the cell cycle and the change of cell proliferation ability. Overexpression or overexpression of miRNA in the tumor may indicate that the expression level of miRNA may affect the type of cell transformation (27). Mir-135 was down-regulated in gastric cancer, renal cancer, prostate cancer and epithelial ovarian cancer. The inhibition may be related to signal transduction, cell cycle progression and the mechanism of p53. Mir-135 can also be used as a carcinogenic gene, such as up-regulating lung cancer, colorectal cancer, breast cancer and other tumors (26-28). It may participate in PTEN / PI3K, hippo, Wnt, DNA methylation and NF - κ B dual regulatory mechanisms (29). Mir-135a can enhance the expression of Caspase-3 and p53, reduce the expression of Bcl-2, promote cell apoptosis, and affect cell growth and adhesion (30). Therefore, mir-135a affects the proliferation and apoptosis of tumor cells by acting on target genes. The expression of mir-135a was down-regulated in tumor cells. When mir-135a was overexpressed in M17 and M21, MTS showed that the proliferation of tumor cells was significantly inhibited (31). The cell cycle consists of four stages: G1, s, G2 and M. the interphase (G1, s, G2) is the material preparation stage (16, 32). The external environment of cells, including nutritional conditions, cell-cell interaction, survival factors, division signals, etc., will affect whether the remaining cells (G0 stage) enter into cell division. One of the most important factors affecting the cell cycle is the regulatory factors at all levels (33).

As the basis of tumor metastasis and diffusion, the migration ability of tumor cells is affected by miRNA (34, 35). When mir-135a was overexpressed in tumor cells M17 and M21, we detected the cell migration ability by Transwell migration test, and found that the cell migration ability of the experimental group through 8 polyacidic M cells was significantly less than that of the control group NC cells; we used RNAi interference technology to interfere with the expression of target gene SIRT1 and found that the migration ability of tumor cells was significantly inhibited. MiRNAs play a key role in caspase, mitochondria and other apoptotic pathways. MiRNAs can regulate the expression of apoptosis pathway protein and induce apoptosis. The expression of these miRNAs in HCC is often disordered. Later, it was found that mir-17-5p and its target protein E2F1 had similar expression patterns in human liver embryogenesis and malignant tumors, and the higher the degree of malignancy, the closer the expression pattern. In the process of embryonic development, the increase of mir-17-5p expression in colon tissue can inhibit the expression of E2F1 and promote cell proliferation. In mature intestinal tissues, the expression of mir-17-5p returned to a low level. In the process of hepatocarcinogenesis, the expression of mir-17-5p has activated again, which led to a malignant proliferation of cells. MiR-143 can regulate the expression of Pk5, NF - κ B and Bcl-2, which suggests that miR-143 may be a tumor suppressor gene-regulating apoptosis. Liu *et al*. (22) and other related studies also found that miR-143 and miR-145 are tumor suppressor genes, and their expression is down-regulated. Extracellular signal-regulated kinase 5 is one of the target genes of miR-143. Zhao *et al*. (10) related protein rab1b regulates vesicular transport between the endoplasmic reticulum and Golgi matrix and directly affects autophagy. Mir-502 was down-regulated in HCC, rab1b was the direct target of mir-502; in HCC, overexpression of mir-502 inhibited the expression of p53 and rab1b, so as to inhibit the autophagy process and the growth of SW480, HCT116 and HCC.

The effect of miRNAs on the signal transduction pathway is also one of the mechanisms of tumor regulation. It was found that the over-expressed miR-145 could act on Wnt/catenin pathway in human colon cancer cells, and interfere with the translocation of catenin to the nucleus by acting on the target gene catenine3-1, thus inhibiting the growth of colon cancer cells (2). In HCC cells, miR-145 expression was significantly down-regulated, which promoted Wnt/catenin pathway and cancer cell growth. The expression of PDCD4 was down-regulated in miR-21 transfected hepatoma cells, while the expression of PDCD4 protein was up-regulated in anti-miR-21 transfected hepatoma cells, and the cell activity and invasiveness were decreased; overexpression of miR-21 could significantly inhibit the expression of PDCD4 at the post-transcriptional level, and increase the invasiveness of cells, suggesting that miR-21 could inhibit the expression of PDCD4 and promote the invasion and metastasis of hepatoma cells. MiR-21 promotes the metastasis of hepatoma cells by inhibiting the expression of PDCD4. The expression patterns of miRNAs are different in different tumors. In order to reveal the inhibitory effect of exogenous resources on the proliferation of hepatoma cells, five tumor suppressor microRNAs can regulate the expression of various factors in the signal pathway, such as karst, catenin δ - 1, and regulate various life activities of tumor cells. Wnt signaling pathway controls cell proliferation, migration and embryonic development. The Wnt signaling pathway is an important factor in the development of liver cancer. In 90% of colorectal cancer, Wnt/catenin pathway is mutation and active. On the contrary, if APC function is lost, tandem protein will not phosphorylate and accumulate in the cytoplasm, enter the nucleus, start the downstream signal and activate Wnt signal pathway.

Based on the results of this study, we believe that mir-135a can inhibit the proliferation of hepatoblastoma cells by regulating the expression of the Notch pathway. Inhibit the proliferation and migration of tumor cells. Mir-135 inhibits tumor cell proliferation by regulating the cell cycle. It can inhibit the proliferation and migration of tumor cells by regulating various signaling pathways.

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None.

Interest conflict

None.

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