MIR-134-5P REGULATES MIGRATION AND INVASION OF LIVER CANCER CELLS THROUGH ITGB8

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ABSTRACT

Objective: *To investigate the effect of miR-134-5p on the migration and invasion of liver cancer (LC) cells through regulating ITGB8.*

Methods: *A total of 68 patients with LC admitted to our hospital from May 2016 to May 2018 were enrolled in the research group (RG), and another 65 healthy subjects with concurrent physical examination were selected as the control group (CG) for prospective analysis. The expression levels of miR-134-5p and ITGB8 in peripheral blood of the two groups were detected, and the clinical value of miR-134-5p in LC was analyzed. In addition, LC cells were purchased for biological function testing.*

Results: *The expression levels of miR-134-5p and ITGB8 in the peripheral blood of the RG were higher than those of the CG (P<0.050). The ROC curve analysis showed that miR-134-5p had a good predictive value for the occurrence of LC. After the transfection of miR-134-5p-mimics (overexpression sequence), the proliferation, invasion and migration ability of LC cells were decreased, and the apoptosis and Caspase-3 and Caspase-9 proteins were increased (P<0.050). While conversely, the transfection of sh-ITGB8 (overexpression sequence) brought markedly elevated proliferation, apoptosis and invasion ability, as well as reduced apoptosis and Caspase-3 and Caspase-9 proteins of LC cells (P<0.050). The dual fluorescein reporter enzyme demonstrated that ITGB8 was the target gene of miR-134-5p.*

Conclusion: *MiR-134-5p was lowly expressed in LC, and it could inhibit the proliferation, migration and invasion of LC cells through targeted regulation of ITGB8.*

Keywords: *MiR-134-5p, liver cancer, ITGB8, migration, invasion.*

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Introduction

Liver cancer (LC) is a common malignant tumor with strong heterogeneity, which is extremely common worldwide[1]. The onset of LC is insidious, and there is usually no obvious special manifestation in the early stage. As a result, the disease has always developed into the middle and late stage once diagnosed[2]. It is estimated that there are more than 1 million newly diagnosed LC patients in the world every year, and in recent years, the number has been increasing year by year[3, 4]. In addition to primary LC, the liver is also a common metastatic site of gastrointestinal malignant tumors, lung cancer, breast cancer, etc., which is extremely important for the human body[5]. At present, the mainstream treatment methods for LC in the clinic are mainly surgery or combined with chemoradiotherapy. However, with the progression of the disease and the decrease of chemotherapy sensitivity, the prognosis of patients is not optimistic, which is also one of the main reasons for the great death threat of LC[6].

According to statistics, the prognosis mortality rate of LC has exceeded 11.5%, which is the second highest mortality rate after gastric cancer and esophageal cancer[7, 8]. Currently, the pathogenesis of LC has not yet been clarified, and understanding the occurrence and development of LC is a key factor for

future clinical diagnosis and treatment of it. Therefore, researchers at home and abroad are constantly striving to explore the main causes of LC[9]. With the deepening of research, more and more studies have pointed out that microRNA (mRNA) may be the very key to the occurrence of tumor diseases. For example, Hur[10] indicated that miR-203 was involved in the occurrence and development of colorectal cancer, while Conickx[11] demonstrated that miR-218-5p was implicated in chronic obstructive pulmonary disease. As a kind of non-coding short RNA of about 22 nt in length, mRNA plays a major role in inhibiting the translation and transcription of target genes by binding to the 3, untranslated regions (UTR) of its downstream target gene mRNA, thereby changing the expression of target genes[12].

Among them, microRNA-134-5p was proposed in previous studies to have abnormal expression in LC[13]. Pan[14] and Liu[15] also showed that miR-134-5p was closely related to pancreatic cancer and kidney cancer. However till now, no studies have confirmed the relationship between miR-134-5p and LC. Hence here, in order to understand the pathogenesis of LC, this study analyzed the expression of miR-134-5p in LC and its influence mechanism, aiming to provide reference and guidance for future clinical diagnosis and treatment of this disease.

Materials and methods

Patient information

Sixty-eight patients with LC admitted to our hospital from May 2016 to May 2018 were assigned in the RG, and another 65 patients with concurrent physical examination were included in the CG for prospective analysis. This experiment had been approved by the Medical Ethics Committee of our hospital, and all the above subjects had signed informed consent. There were no significant differences in clinical general data (age, gender, body bass index (BMI), etc.) between the two groups (P>0.050).

Inclusion and exclusion criteria

Inclusion criteria:

• Patients aged 30 to 70 years old, in line with the clinical manifestations of LC, who were confirmed by the Department of Pathology biopsy and received follow-up treatment in our hospital after diagnosis of LC, with complete case data. All the patients agreed to participate in the investigation of our hospital, and did not receive any adjuvant treatment before admission.

Exclusion criteria:

• Patients complicated with other tumors, heart, brain and blood diseases, chronic diseases, mental diseases, autoimmune diseases;

• Patients with organ failure;

• Patients with hepatic and renal insufficiency;

• Patients allergic to the drugs used in this study;

• Patients with long-term physical disability bedridden and unable to take care of themselves;

• Patients transferred from other hospitals;

• Patients died during treatment.

Inclusion and exclusion criteria of the CG:

• Those aged 30-70 years, without any disease, whose physical examination results showed that all the tests were normal, and agreed to participate in the investigation of our hospital.

Cell data

LC cells H22 (BNCC338327), HLE (BNCC100966), Huh-7 (BNCC337690), BEL-7402 (BNCC338237) and human normal hepatocyte HL-7702 (BNCC351907) were all purchased from ATCC. The culture was carried out in a DMEM medium containing 10% PBS, 2 mm penicillin and streptomycin at 37 ° C and 5% CO_2.

Main reagents

QRT-PCR and reverse transcription kit (TransGen Biotech, Beijing, China AQ201-01, AQ202-01), CCK-8 kit (Promega, USA), Transwell kit (Shanghai Fanke Biotechnology Co., Ltd., FK-lk019) , PBS, fetal bovine serum (FBS) (Gibco, USA 10010049, 10437028), Trizol reagent (Beijing Biolab Technology Co., Ltd., QN2070-ZOG), dual luciferase reporter gene detection kit (Beijing Biolab Technology Co., Ltd., KFS303-TFX), RIPA, BCA Protein Kit (Thermo Scientific, USA), Annexin V-FITC/PI Apoptosis Kit (Beijing Jiamay Biotechnology Co., Ltd., LHK601-020).

ITGB8, Caspase-3, Caspase-9 and β-Actin antibody (Cell Signaling Technology Company), goat anti-rabbit IgG secondary antibody (Wuhan Boster Biological Technology Co., Ltd.), ECL developer (Thermo), PCR instrument (ABI, USA, 7500). All primers were designed and synthesized by Shanghai Sangon Biotechnology Co., Ltd.

PCR

Trizol reagent was applied to extracted the total RNA from tissues and cells, 5 μg of which was then taken for reverse transcription of cDNA according to the kit instructions.

After that, 1 μL of synthetic cDNA was amplified for amplification: cDNA: 1 μL, upstream and downstream primers: each 0.4 μL, 2X TransScript® Tip Green qPCR SuperMix: 10 μL, Passive Reference Dye (50X): 0.4 μL, and Nuclease-free Water was added to complete the reaction volume of 20 μL. With U6 as the internal reference of miR-134, and β-Actin as that of ITGB8, the data was analyzed by using $2^{-\triangle\triangle ct}$.

	Forward (5,-3,)	Reverse (5,-3,)
miR-134-5p	GGTGTGACTGGTTGACCA	TGCGTGTCGTGGAGTC
U6	CTCGCTTCGGCAGCACATA	GTGCAGGGTCCGAGCT
ITGB8	TTTTGAGCTCGTACTGCCCG TGCAAATCCCACAA	TTTTACGCGTTGCTTTTCCT CAACTTCTTTAA
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

Table 1: Primer sequence.

Western blot

The cells were lysed by RIPA lysis and total protein was extracted, and then the protein concentration was measured by the BCA assay. Adjusted the protein concentration to 4 μg/μL, electrophoretically separated by 12% SDS-PAGE before transferring to PVDF membrane, and then sealed with 5% skim milk powder for 2h. Next, ITGB8 (1:500), Caspase-3 (1:500), Caspase-9 (1:500), and β-Actin (1:1000) primary antibody were added and blocked overnight at 4 °C. After that, the primary antibody was washed and the horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1000) was added, incubated at 37 °C for 1h, and rinsed 3 times with PBS, 5 min each.

Finally, development was carried out in a dark room, the excess liquid on the film was firstly blotted with a filter paper, and then illuminated by ECL to develop. The protein bands were scanned and the gray value was analyzed using Quantity One software, wherein the relative expression level of the protein = the gray value of the target protein band/ the gray value of the β-Actin protein band.

Cell transfection

When the cell adherent growth and fusion reached 85%, 25% trypsin was added for digestion. After that, it was placed into the culture medium for further culture and passage.

Upon completion, the expression of miR-134 in each cell line was detected, and the ones with the greatest difference from normal liver cells were selected for subsequent transfection. Transfection of miR-134-5p-mimics (overexpression sequence), miR-134-5p-inhibitor (inhibition sequence), miR-NC (negative control), sh-ITGB8 (target overexpression), si-ITGB8 targeted inhibition), and NC (negative control) were performed with Lipofectamine™ 2000 kit, and the procedures were strictly in accordance with the kit instructions.

CCK-8

Cells were collected 48h after transfection, diluted into 3×104cell/ml, and seeded in 96-well plates (100μl cells per well), and cultured at 37°C with 5% CO2. At 0h, 24h, 48h and 72h after cell adherent growth, 10μl CCK8 solution was added to each well and continued to culture in an incubator with 5% CO2 at 37°C for 2h.

The OD value was then measured at 450 nm using a microplate reader to detect cell proliferation and draw a growth curve. The experiment was repeated three times.

Flow cytometry

The transfected cells were digested with 0.25% trypsin, washed twice with PBS, added 100μL binding buffer, and configured into $1^{*}106$ /mL suspension. Followed by the successive addition of Annexin V-FITC and PI, incubated at room temperature for 5 minutes in the dark, and finally detected by FACSVerse flow cytometry system. The experiment was repeated 3 times for average value.

Transwell

An volume of 200 μL DMEM medium containing 1×105 cells was added to the upper chamber, while the lower chamber was added with 500 mL DMEM containing 20% FBS, then cultured at 37° C for 48 hours.

The matrix and cells of the upper chamber that failed to cross the membrane surface were wiped, washed three times with PBS, and fixed with paraformaldehyde for 10 minutes.

Followed by a triple rinse with double distilled water and then stained with 0.1% crystal violet after drying. The invasion of cells was observed under a microscope.

Wound-healing assay

The cells were dissected using a 200 μl sterile pipette tip to form a cell-free area, and the squashed cells were washed with PBS and added to a new medium for culture. The cell migration ability was eval-

uated by microscopy of scratches at three different locations at 0h (W0) and 24h (W24) after the cells were scratched.

Dual luciferase assay

The ITGB8-3,UTR wild type (Wt) and ITGB8-3, UTR mutant (Mut) and miR-134-5p-mimics and miR-NC were transfected into LC cells using LipofectamineTM 2000 kit after transfection. Luciferase activity was measured 48 hours after transfection by a dual luciferase reporter assay kit (Promega).

Statistical methods

The data were analyzed and processed by SPSS22.0, and plotted by GraphPad8.0. The counting data were expressed in the form of percentage, and the Chi-square test was employed for inter-group comparison. While the measurement data were expressed as mean ± standard deviation.

The inter-group comparison was performed by the t-test, and the inter-group comparison was conducted by one-way ANOVA and LSD post-test. Cell performance at multiple time points were compared using repeated measures analysis of variance and Bonferroni post-hoc test. The diagnostic value was analyzed by ROC curve. The correlation was analyzed by Pearson correlation coefficient.

The survival rate was calculated by kaplan-meier method, and the survival rate was compared by Log-rank test. COX regression analysis was performed for risk factors. A statistically significant difference was assumed at P<0.050.

Results

Clinical value of miR-134-5p in LC

The miR-134-5p in the peripheral blood of the RG was significantly lower than that of the CG (P<0.050).

The ROC curve analysis showed that when the cut-off value was 0.695, the predictive sensitivity of miR-134-5p in peripheral blood for detection of LC was 85.29%, the specificity was 63.08%, the AUC was 0.803, and the 95% CI was 0.730-0.877. In the RG, miR-134-5p was found to be significantly lower in cancer tissues than that in paracancer tissues (P<0.050), while ITGB8 was significantly higher (P<0.050).

According to Pearson correlation coefficient analysis, miR-134-5p was negatively correlated with ITGB8 in cancer tissues (r=-0.650, P<0.001). (Figure 1).

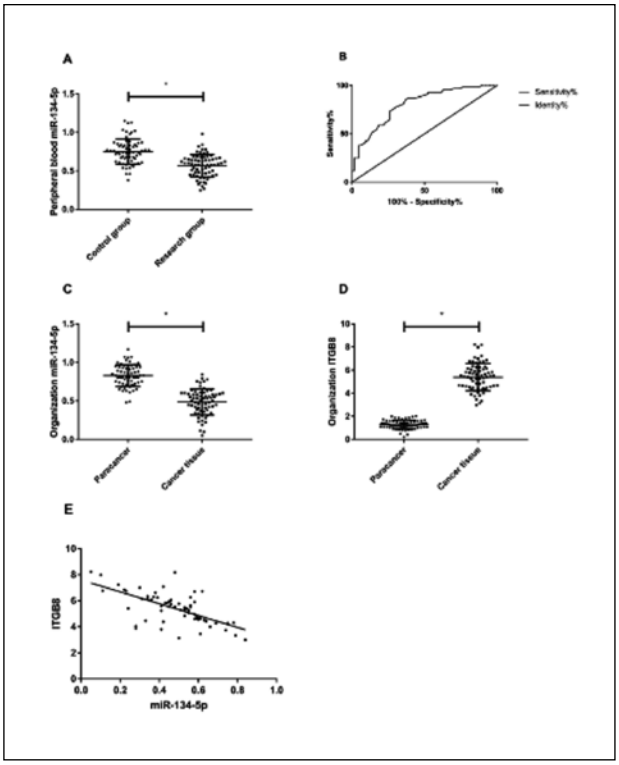

Figure 1: Clinical value of miR-134-5p in LC .
*A) Comparison of miR-134-5p expression levels in peripheral blood of the two groups, * indicates P<0.050; B) ROC curve analysis of miR-134-5p in peripheral blood on the occurrence of LC; C) Comparison of miR-134-5p expression level between cancer tissue and paracancerous tissues in the RG, * indicates P<0.050; D) Comparison of ITGB8 expression level between cancer tissue and paracancerous tissues in the RG, *indicates P<0.050; E) Correlation analysis of miR-134-5p and ITGB8 in cancer tissues.*

Effects of miR-134-5p on the prognosis of LC

We performed a one-year prognosis follow-up for patients with LC, of which all these 68 patients were successfully followed up, with a follow-up success rate of 100.0%. According to the expression level of miR-134-5p in peripheral blood of patients, we divided the patients into group A (miR-134-5p<0.57, n=39) and group B (miR-134-5p≥0.57, n=29). The prognosis and survival curves of the two groups showed that the prognosis of the patients in group A was significantly worse than that in group B (P < 0.050). Meanwhile, we observed the expression of miR-134-5p in the peripheral blood of the dead and the surviving patients, and found that miR-134-5p in the dead patients was significantly lower than that in the surviving patients (P<0.050).

The ROC curve analysis exhibited that when the cut-off value was 0.675, the sensitivity of peripheral blood miR-134-5p in predicting the death of within 1 year was 66.67%, the specificity was 87.50%, the AUC was 0.838, and the 95% CI was

0.728-0.948, P<0.001. And according to COX analysis, pathological stage, TNM staging, degree of differentiation, lymphatic metastasis and miR-134-5p were independent factors affecting the prognosis of patients with LC (P<0.050). (Figure 2, Table 2).

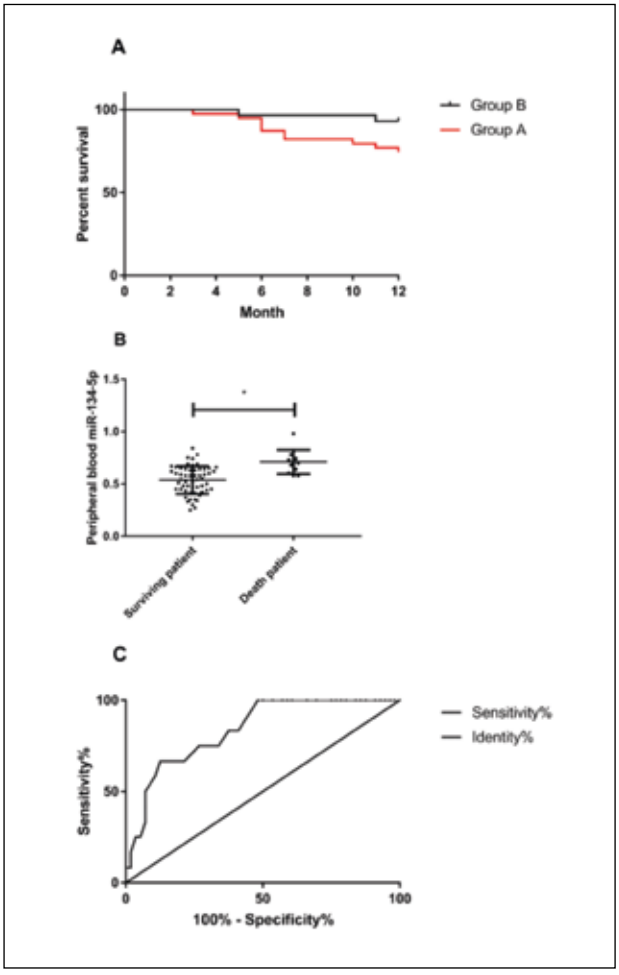

Figure 2: Effects of miR-134-5p on the prognosis of LC. *A) 1-year survival curve of prognosis of group A and group B; B) Comparison of miR-134-5p expression levels in peripheral blood of patients with death and survival, * indicated P<0.050; C) ROC prediction of miR-134-5p in peripheral blood for prognosis and death of patients.*

Variables	Univariate analysis			Multivariate analysis		
	P	HR	95%CI	P	HR	95%CI
Gender (male vs female)	0.415	0.716	0.342-1.518			
Age (<60 years old vs ≥60 years old)	0.486	0.763	0.384-1.605			
Pathological stages (I-II vs III-IV)	0.016	2.516	1.314-4.485	0.021	3.032	1.041-6.614
Lymph node metastasis (with vs without)	0.009	2.762	1.421-4.623	0.012	2.515	1.312-4.627
Differentiation degree (low vs moderate and high)	0.025	2.052	1.262-5.317	0.006	2.869	1.645-4.019
miR-134-5p (<0.57vs≥0.57)	0.005	4.354	1.562-7.426	0.008	3.651	1.842-4.261

Table 2: COX analysis.

Effects of miR-134-5p on LC cells

The expression level of miR-134-5p in LC cell lines H22, HLE, Huh-7, BEL-7402 were detected to be lower than that of human normal hepatocyte HL-7702 (P<0.50), among which the expression level of miR-134-5p in H22 and HLE were the lowest. Therefore, these two were selected for subsequent experiments. Then the biological behavior detection was conduction by transfecting miR-134-5p into H22 and HLE cell lines. It was found that miR-134-5p-mimics group had significantly decreased cell proliferation, invasion and migration ability, and elevated apoptosis ability and Caspase-3, Caspase-9 protein (P<0.050), while conversely, the proliferation, invasion and migration ability of miR-134-5p-inhibitor group was significantly increased, and the apoptosis ability and Caspase-3 and Caspase-9 protein were significantly decreased (P<0.050). (Figure 3).

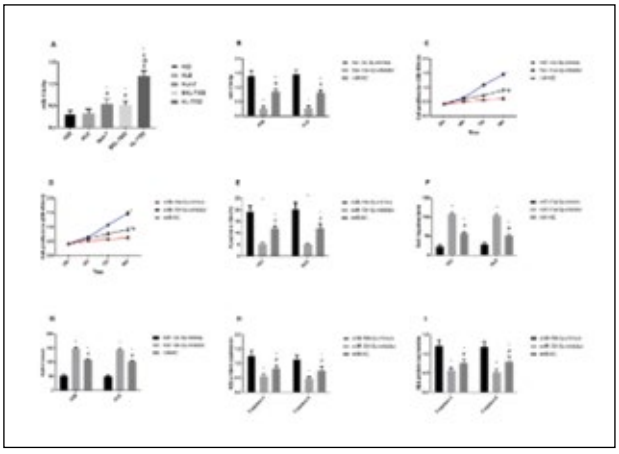

Figure 3: Effects of miR-134-5p on LC cells. *A) Expression of miR-134-5p in LC cells and normal liver cells; B) Expression level of miR-134-5p after transfection; vC) Proliferation ability of H22 cells; vD) Proliferation ability of HLE cells; vE) Apoptosis rate; vF) Cell migration ability; G) Cell invasion ability; H) Expression of H22 cell protein; I) Expression of HLE cell protein.*

Effects of ITGB8 on LC cells

The detection of expression levels of ITGB8 in LC cell lines H22, HLE, Huh-7, BEL-7402 and human normal hepatocyte HL-7702 revealed that ITGB8 was remarkably increased in LC cells (P<0.050). After transfecting ITGB8 into H22 and HLE cell lines, the biological behavior of sh-ITGB8 cells was significantly increased, and the apoptosis ability and Caspase-3 and Caspase-9 protein were decreased (P<0.050). While the cell proliferation, apoptosis and invasion ability of si-ITGB8 group were significantly decreased, and the apoptosis ability and Caspase-3 and Caspase-9 protein were increased (P<0.050). (Figure 4).

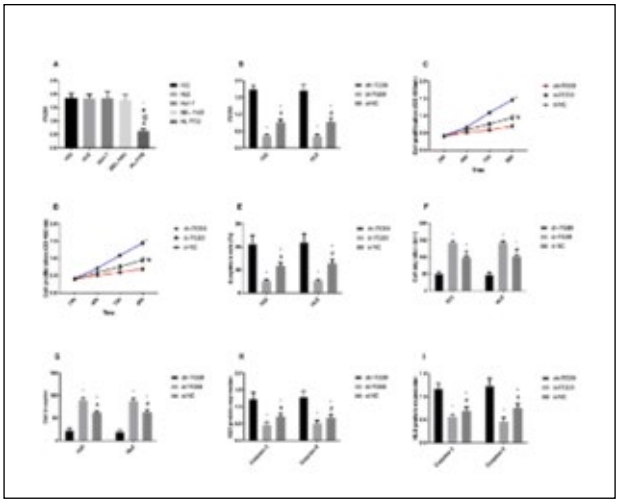

Figure 4: Effects of ITGB8 on LC cells.
A) Expression of ITGB8 in LC cells and normal liver cells; B) Expression level of ITGB8 after transfection; C) Proliferation ability of H22 cells; D) Proliferation ability of HLE cells; E) Apoptosis rate; F) Cell migration ability; G) Cell invasion ability; H) Expression of H22 cell protein; I) Expression of HLE cell protein.

Correlation between miR-134-5p and ITGB8

To further verify the relationship between miR-134-5p and ITGB8, we first predicted that there were targeted binding sites between the two through predicting the downstream target gene of miR-134-5p by Targetscan7.2.

The double luciferase activity assay demonstrated that the luciferase activity of pmirGLO-ITGB8-3,UT Wt was significantly decreased after miR-134-5p overexpression ($P<0.05$), but to that of pmirGLO-ITGB8-3,UTR Mut, there was no significant effect ($P>0.05$).

The WB detection revealed that the expression of ITGB8 protein in H22 and HLE cells was significantly higher than that in miR-134-5p-mimics group after transfection with miR-134-5p-inhibitor ($P<0.05$). (Figure 5).

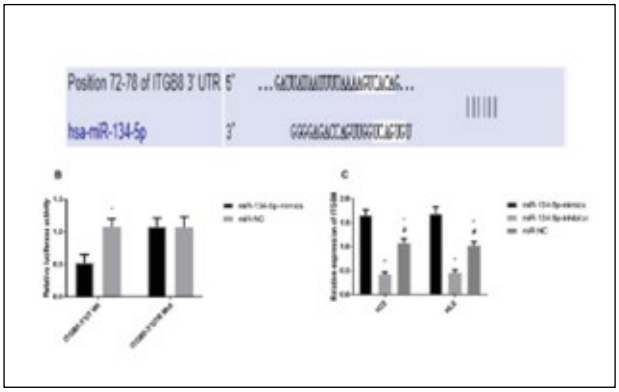

Figure 5: Correlation between miR-134-5p and ITGB8.
A) Dual luciferase reporter; B) ITGB8 protein expression after transfection of miR-134-5p.

Discussion

As one of the malignant tumors that are threatening the human body in clinical practice, LC has continuously increased its morbidity and mortality[16]. At present, the pathogenesis of LC is not clear, but studies have indicated that intervention through the pathogenic factors at the beginning of LC may be a major breakthrough in the future treatment of LC[17]. With the gradual clinical confirmation of the relationship between miR and tumor diseases, the role of miR as a diagnosis and treatment marker of tumor diseases has become a research hotspot in clinical practice[18, 19]. Though currently, the effect of miR-134-5p on LC has not been clarified, this study is of great significance for future clinical diagnosis and treatment of LC by analyzing the effect of miR-134-5p on LC and exploring its mechanism.

The results of this study showed that miR-134-5p was lowly expressed in peripheral blood and cancer tissues of patients with LC, while ITGB8 was highly expressed, suggesting that miR-134-5p and ITGB8 may be involved in the occurrence or development of LC. This is also consistent with the study of miR-134-5p and ITGB8 in nasopharyngeal carcinoma and glioma conducted by Liu Y[20] and Li[21], which can support our experimental results. Through ROC curve analysis, we found that miR-134-5p had a predictive sensitivity of 85.29% for LC and a specificity of 63.08%, suggesting that miR-134-5p could be used as an early diagnostic marker for LC to assist clinical detection. Compared with traditional tumor markers, miR-134-5p has higher specificity and is more advantageous for identifying LC. Moreover, COX analysis also validated that miR-134-5p was an independent factor affecting the prognosis of patients, further confirming the clinical application value of miR-134-5p in LC. Studies displayed that miR-134-5p was involved in the occurrence of high glucose-induced podocyte apoptosis through bcl-2[22], and studies by Chang[23] also indicated that miR-134-5p regulated RAB27A and affected the development of ovarian cancer.

To further clarify the influencing mechanism of miR-134-5p on LC, we conducted in vitro experiments to improve our results. By detecting the expression of miR-134-5p in LC cells and normal hepatocytes, we found that miR-134-5p also presented low expression in LC cells which could also verify the accuracy of our above results. The detection of biological behavior of LC cells transfected with miR-134-5p indicated that the proliferation,

invasion and migration of LC cells were significantly reduced after the overexpression of miR-134-5p, and the apoptosis ability was increased, while the inhibited expression of miR-134-5p resulted in significantly increased growth ability of LC cells, suggesting that miR-134-5p plays a role as a tumor suppressor gene in LC. This is also in line with Liu Q,s study[24] on the influencing mechanism of miR-134-5p on non-small cell lung cancer, which supported our results. Previous studies suggested that miR-134-5p had a potential link with ITGB8[25], and a review of relevant literature found that ITGB8 was also abnormally expressed in LC[26].

Therefore, we hypothesized that the mechanism of miR-134-5p affecting LC may be achieved through ITGB8. Further transfecting ITGB8 into LC cells revealed that the proliferation, invasion and migration ability of LC cells increased significantly after overexpressing ITGB8, which also confirmed that ITGB8 was closely related to the occurrence and development of LC. Further, we predicted the downstream target gene of miR-134-5p by Targetscan7.2, and found the target binding sites between ITGB8 and miR-134-5p. The dual luciferase activity assay was then carried out, whose results showed that the miR-134-5p overexpression significantly decreased the prmGLO-ITGB8-3,UT Wt luciferase activity, confirming that ITGB8 is a target gene of miR-134-5p. Apart from that, the protein expression of IGBG8 in the miR-134-5p inhibitory sequence was detected to be significantly elevated, so we determined that miR-134-5p inhibited the progression of LC by targeting ITGB8. This experiment still has some shortcomings due to limited experimental conditions. For example, we failed to test the drug resistance of miR-134-5p, which in turn resulted in the failure of determining the impact of miR-134-5p on the drug resistance of LC patients. What,s more, because of the short experimental period, the long-term prognosis of miR-134-5p on LC patients cannot be assessed. Moreover, patients with benign disease liver disease were excluded in this study, so the exact value of miR-134-5p as a diagnostic marker for LC could not be further verified. Nevertheless, more in-depth and comprehensive experimental analysis will be carried out in the future to address the above shortcomings, so as to obtain more representative experimental results for clinical reference.

In summary, miR-134-5p is lowly expressed in LC, and its role as a tumor suppressor gene by targeting ITGB8 may be a potential target for the diagnosis and treatment of LC in the future.

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