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Effect of microRNA-7 on proliferation, invasion, migration and EMT of hepatoma cell line SMMC-7721

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Abstract. – OBJECTIVE: This study aimed to observe the effect of microRNA-7 on the proliferation and migration of human hepatocellular carcinoma cell line SMMC-7721 and its effect on epithelial-mesenchymal transition (EMT).

PATIENTS AND METHODS: Eukaryotic expression vector pcDNA3.1(-)-pri-miR-7(p-miR-7) was used to instantaneously transfect human liver cancer cells of SMMC-7721 cells *in vitro*. The expression of microRNA-7 was detected by RT-qPCR. Western blot was used to detect the expression of EMT marker proteins E-cadherin, β-catenin, N-cadherin and Vimentin. The proliferation of SMMC-7721 cells was detected by CCK-8 assay, and the invasion and migration ability of cells was detected by transwell assay.

RESULTS: Compared with the normal group, the expressions of E-cadherin and β-catenin in SMMC-7721 cells transfected with miR-7 were significantly increased ($p<0.05$), while the expressions of N-cadherin and Vimentin were significantly decreased ($p<0.05$). Meanwhile, the proliferation, invasion and migration ability of the cells were significantly weakened ($p<0.05$).

CONCLUSIONS: The miR-7 can inhibit the proliferation and invasion of human hepatocellular carcinoma cell line SMMC-7721, and its mechanism may be related to upregulation of E-cadherin, β-catenin protein, and downregulation of N-cadherin and Vimentin proteins.

Key Words:
MicroRNA-7, SMMC-7721, Proliferation, Invasion, EMT.

Introduction

Globally, hepatocellular carcinoma (HCC) is the fifth most common cancer among men, the ninth most common cancer among women, and the second most common cause of cancer death[1,2]. Currently, surgical interventions, such as liver resection, liver transplantation and percutaneous ablation are considered to be the most effective methods for the treatment of HCC, with therapeutic potential. Unfortunately, due to multiple lesions and extrahepatic metastases, only about 20% of HCC patients are suitable for surgery. Moreover, the efficacy of HCC chemotherapy drugs are limited. For example, sorafenib, as one of the most common prescriptions, its clinical benefit is limited, only effective for around 30% of the patients, and there would be resistance in 6 months. In addition, the current treatment methods have problems, such as hepatotoxicity, recurrence, drug resistance and other adverse reactions. Due to the difficulty in early diagnosis, rapid progress and lack of targeted drugs, the 5-year survival rate of HCC patients is estimated to be less than 9%[3,4]. Therefore, it is necessary to further study the pathogenesis of HCC and find targeted molecular therapeutic targets, in order to provide new therapeutic directions, improve the therapeutic effect and prolong the survival time for HCC patients.

MicroRNAs (miRNA) are a class of short (21-22 nt) endogenous non-coding RNAs. They mediate the post-transcriptional regulation of gene expression and play the role of guiding molecules in the process of RNA silencing. They are important regulators of gene expression[5-7]. In the regulation process of various oncogenes or tumor suppressor genes, their expression profiles have been clinically used as a biomarker of diagnosis and prognosis. They could be used to evaluate the occurrence, development of tumors and the response to treatment of cancer patients[8]. MiR-7 is a 23-nucleotide miRNA[9]. MiR-7 plays an important role in the growth,

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migration and invasion of prostate cancer[10]. Hu et al[11] indicate that it can also significantly inhibit the growth of lung cancer cells. However, there are few researches on the role of miR-7 in HCC clinically, which is worthy of further investigation.

Therefore, this study explored the effect of miR-7 on the proliferation, invasion and migration of SMMC-7721 and the epithelial-mesenchymal transition (EMT), in order to provide a reference for clinical practice.

Patients and Methods

Research Object

Thirty HCC patients admitted from June 2016 to December 2017 were enrolled as study group, and 30 healthy patients during the same period were collected as control group. In the study group, there were 20 males and 10 females aged 40-65 years. In the control group, there were 18 males and 12 females aged 40-65 years. Inclusion criteria: patients diagnosed with HCC by preoperative or intraoperative pathology; no radiotherapy, chemotherapy or immunotherapy was given before surgery; all patients received treatment in Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology; clinical data were complete. Exclusion criteria: patients with severe cardiac and renal dysfunction, systemic diseases of blood, mental diseases, other malignant tumors, neurological diseases, systemic autoimmune diseases. The study was approved and filed by the Ethics Committee of Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, and the subjects signed a full informed consent form.

Detection Method

5 mL fasting venous serum of the two groups was collected and analyzed in the morning of the next day after admission. The serum was let stand for 30 min after procoagulant, centrifuged at 4°C, 3500 r·min-1 for 5 min, and was taken and stored in a 1.5 mL centrifuge tube at -80°C refrigerator for testing.

Human hepatoma cell line SMMC-7721 was purchased from the Institute of Cell Research, Shanghai Academy of Health Sciences, China. SMMC-7721 cells were placed in Roswell Park Memorial Institute-1640 (RPMI-1640; Jiehui Bogao Biotechnology Co., Ltd., Beijing, China) medium containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), cultured in a 37°C, 5% CO_2 cell incubator. Then, the cell culture medium was extracted, placed in a centrifuge tube, and centrifuged at 1000 rpm for 5 minutes. The solution was cleared, an appropriate amount of fresh medium was added, fully mixed, and transferred to a new medium for dilution according to the dilution ratio. The cells were divided into three groups, namely, the miR-7 mimics group, the negative control group, and the blank control group.

The logarithmic growth phase SMMC-7721 cells were selected and washed one time with PBS. The 5×10^4 cells were resuspended in RPMI-1640 medium, and then, transferred to the culture medium with 5% CO_2 at 37°C in 24 plate wells. After 12 hours, pcDNÃ3.1(-)-pri-miR-7 (p-miR-7) was transfected into cells according to the instructions of FuGENE-HD kit.

After RT-qPCR Detection

Total RNA was extracted from serum and cultured cells using TRIzol extraction kit (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were detected by Nano-Drop 2000 UV spectrophotometer (Beijing Keyi Xingye Technology Development Co., Ltd., China). RNA was reversely transcribed into cDNA according to TaKaRa Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was stored at -20°C for later use. The primers were designed and synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd. The reaction was performed on ABI PRISM 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). The PCR amplification cycle conditions were as follows: 5 min at 90°C, 5 s at 90°C, 30 s at 60°C, 5 s at 72°C, for a total of 40 cycles. Each sample was repeatedly tested 3 times, and the relative expression of the gene was expressed by $2^{-\Delta CT}$.

Cell Counting Kit-8 (CCK-8) Assay for Cell Proliferation

The cells were diluted to a cell suspension with a density of 5×10^5 /mL, and 100 μL of the cells were inoculated into a 96-well plate. Three Rewells were made in each group, and the cells were 1×10^3/well. The cells were incubated in an incubator at 37°C, and CCK-8 values were measured at 1 d, 2 d, 3 d and 4 d. The original medium was discarded during the detection, and the CCK-8 solution was placed at a ratio of 1:10 by volume (100 μL/well of the test solution was added to the well to be tested, and cultured for 1 hour). The absorbance (OD) values of each well were measured at the wavelength

of 450 nm on Elx-800 enzyme-linked immunosorbent assay (BioTek, Winooski, VT, USA). All samples were repeatedly measured 3 times and the average value was calculated.

Western Blot Analysis of EMT Marker Protein Expression

The cells were trypsinized with 0.25% EDTA, resuspended in serum-free Dulbecco's Modified Eagle's Medium (DMEM; Baienwei Biotechnology Co., Ltd., Shenzhen, Guangdong, China). The cell density of 10^4 cells per well was added to the upper chamber of the transwell chamber in which the Matrigel (Thermo Fisher Scientific, Carlsbad, CA, USA) was pre-plated. 500 ml of the complete culture solution was added to the lower chamber, and cultured in a 37°C, 5% CO_2 cell incubator for 24 hours. The upper chamber was taken out, fixed in 4% paraformaldehyde for 15 min, crystal violet stained for 1 min, and washed with distilled water for 3 times for 3 min each time. The number of transmembrane cells was counted under a 200-fold optical microscope, and five fields of view were randomly selected.

Western Blot Analysis of EMT Marker Protein Expression

The total protein of the cells was extracted from RIPA lysate and quantified by PCA, followed by sample addition, electrophoresis and mold transfer at the rate of 50 μg/well. After the mold transfer, it was sealed with 5% skim milk powder at room temperature for 1.5 h and washed with TBST for 3 min. Each protein antibody was operated according to the antibody specification, incubated overnight at 4°C, and washed with TBST membrane for 3 times, 10 min each time. Enhanced chemiluminescence (ECL) and development were performed. GAPDH was used as an internal reference to analyze the relative expression levels of each index.

Observation Indicators

The general data of the two groups were collected. The expression of miR-7 in the serum of the two groups was compared. The relationship between the expression of miR-7 in the serum of the study group and clinicopathological features was observed. The transfection efficiency of miRNA-7 was compared. The effect of miRNA-7 on proliferation and invasion of SMMC-7721 was observed. The effect of miR-7 on the expression of EMT E-cadherin, β-catenin, N-cadherin and Vimentin was observed.

Statistical Analysis

The Statistical Product and Service Solution (SPSS) 19.0 (IBM Corp., Armonk, NY, USA) was used. The measurement data were expressed as n (%), and the ratio between the two groups was compared using the χ^2-test. The count data were expressed as mean ± standard deviation (mean±SD). The comparison between the two groups was performed by independent sample *t*-test. The comparison among multiple groups was analyzed by ANOVA. Repeated measurement ANOVA was used for the comparison of different time points within the group. LSD test was used for the post-hoc test. $p<0.05$ was considered statistically significant.

Results

General Information

There were 30 cases in the control group and 30 cases in the study group. There was no significant difference in age, sex, smoking and drinking behavior between the two groups ($p>0.05$). There were 16 patients (53.33%) with the tumor in the left lobe of the liver, while 14 patients (46.67%) with the tumor in the right. The AFP, AST, ALT and TBiL in the study group were significantly higher than those in the control group ($p<0.05$). See Table I for details.

Expression of MiR-7 in Serum of Two Groups

The results of RT-qPCR showed that the relative expression levels of miR-7 in the study group and the control group were (1.38±0.68) and (6.34±1.88), respectively. The relative expression of miR-7 in the study group was significantly lower than that in the control group ($p<0.05$) (Figure 1).

The Relationship Between the Expression of MiR-7 in Serum and the Clinicopathological Characteristics of the Study Group

The clinical pathological data of the study group and the expression of miR-7 in the serum of patients were analyzed. It was found that the expression of miR-7 was not correlated with age, gender and tumor location ($p>0.05$), but correlated with the mean tumor diameter (cm), pathological classification, lymph node metastasis and TNM stage ($p<0.05$), as shown in Table II.

Table I. General information.

	Experimental group (n=30)	Control group (n=30)	χ^2/t	p
Age (year)	50.36±10.35	48.63±12.63	0.580	0.564
Gender [n(%)]			0.287	0.592
Male	20 (66.67)	18 (60.00)		
Female	10 (33.33)	12 (40.00)		
BMI	22.85±2.36	21.36±2.69	2.281	0.026
Smoke [n(%)]			0.082	0.775
Yes	22(73.33)	21(70.00)		
No	8(26.67)	9(30.00)		
Drink [n(%)]			0.635	0.426
Yes	20 (66.67)	17 (56.67)		
No	10 (33.33)	13 (43.33)		
Tumor location [n(%)]			—	—
Left	16 (53.33)	—		
Right	14 (46.67)	—		
AFP (ng/ml)	252.24±35.36	8.42±2.36	37.684	<0.001
AST (μ/L)	103.55±25.33	15.36±5.69	18.606	<0.001
ALT (μ/L)	113.68±20.96	17.63±8.31	23.333	<0.001
TBiL (μmol/L)	32.64±6.64	9.25±3.61	16.951	<0.001

Expression of MiR-7 in SMMC-7721 Cells After Transfection

The relative expression level of miR-7 in control group in SMMC-7721 cells was (1.26±0.57), the negative control group was (1.23±0.58), and the miR-7mimics group was (8.52±2.65). There was no significant difference between the blank control group and the negative control group, but the miR-7mimics group was significantly higher than that in the other two groups (p<0.05), indicating that transfection was effective (Figure 2).

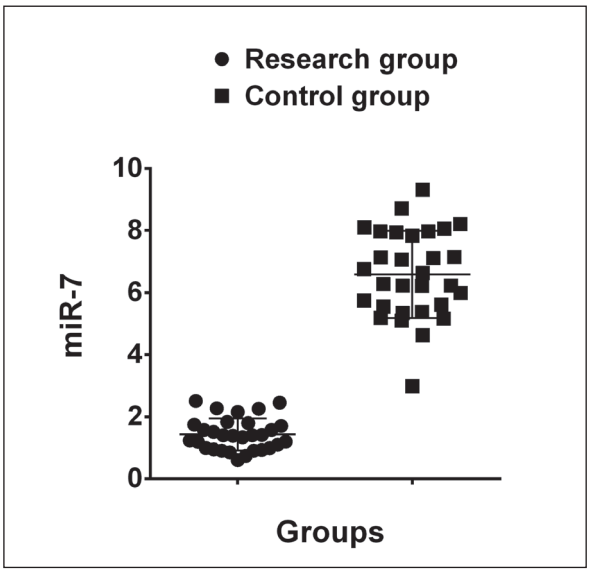

Figure 1. Expression of miRNA-7 in serum from two groups of people. The relative expression of miR-7 in the study group was significantly lower than that in the control group, and the difference was statistically significant (p<0.05).

Effect of MiR-7 on Invasion of SMMC-7721

After transfection and culture for 24 hours, the number of cells that penetrated into the micropore membrane of the compartment was (63.21±8.32) in the miR-7 mimics group, (113.51±12.65) in the blank control group, and (110.63±13.51) in the negative control group. The number of invasion in the miR-7 mimics group was significantly lower than that in the other two groups, and the difference was statistically significant (p<0.05). There was no significant difference in the number of invasion between the blank control group and the negative control group (Figure 3).

Effect of MiR-7 on the Proliferation of SMMC-7721

There was no significant difference in the proliferation of the three groups at 1 d and 2 d. At 3 d and 4 d, the cell proliferation of the miR-7 mimics group was significantly lower than that of the blank control group and the negative control group, and the difference was statistically significant (p<0.05). There was no significant difference between the blank control group and the negative control group at any time point (p>0.05) (Figure 4).

Table II. Relationship between expression of miR-7 in serum of patients in study group and clinicopathological features.

	No. of people	Relative expression of miR-7	χ^2/t	p
Age (year)			0.156	0.887
≤55	17 (56.67)	3.21±0.82		
>55	13 (43.33)	3.16±0.93		
Gender [n (%)]			0.166	0.870
Male	20 (66.67)	3.12±1.12		
Female	10 (33.33)	3.05±1.03		
Tumor location [n (%)]			0.075	0.941
Left	16 (53.33)	3.10±1.14		
Right	14 (46.67)	3.13±1.05		
Mean tumor diameter (cm)			3.656	0.001
≤5	17 (56.67)	3.76±0.86		
>5	13 (43.33)	2.63±0.81		
Pathological type [n (%)]			3.340	0.002
well-differentiated	15 (50)	3.64±0.87		
Middle and low differentiation	15 (50)	2.51±0.98		
Lymphatic metastasis [n(%)]			3.338	0.002
Yes	17 (56.67)	2.46±0.86		
No	13 (43.33)	3.55±0.92		
TNM staging [n (%)]			2.569	0.016
I-II	16 (53.33)	3.54±0.97		
III-IV	14 (46.67)	2.56±1.12		

Effects of MiR-7 on the Expression of E-Cadherin, β-Catenin, N-Cadherin and Vimentin

Compared between the blank control group and the negative control group, the expressions of E-cadherin and β-catenin in SMC-7721 cells transfected with miR-7 in miR-7 mimics group were significantly increased (p<0.05), while the expressions of N-cadherin and Vimentin were significantly decreased (p<0.05). There was no significant difference between the blank control group and the negative control group (Figure 5).

Discussion

HCC is one of the most common malignancies. According to global epidemiological statistics, the number of people dying from liver cancer each year is more than 700,000, and the number of deaths is increasing every year. Meanwhile, the age of onset is getting younger[12,13]. Hepatectomy is the most commonly used and most effective method for the treatment of HCC liver cancer[14]. Its 5-year survival rate is about 30%-40%[15], and the survival rate is lower for patients who meet the surgical criteria. Therefore, it is necessary to further explore the pathogenesis of HCC and targeted molecular therapeutic targets to provide new therapeutic directions. miRNA is a kind of small non-coding RNA that plays a central role as a major regulator of gene expression in a variety of cancer-related signaling pathways[16]. The cell

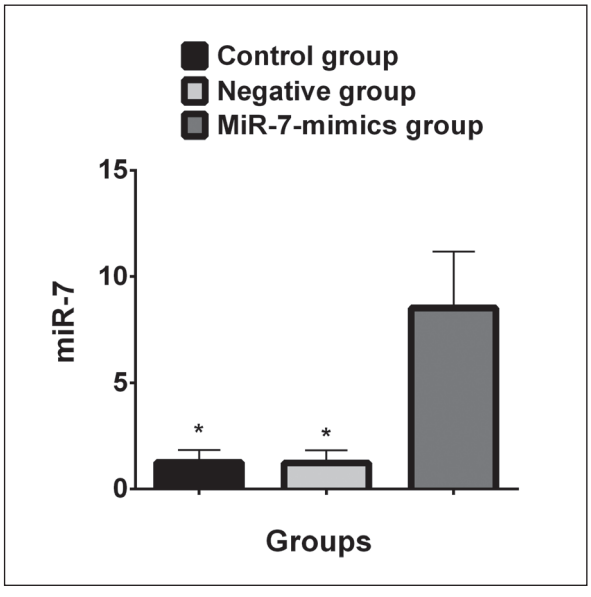

Figure 2. Expression of miRNA-7 in SMMC-7721 cells post transfection. The expression of miR-7 in the miR-7mimics group was significantly higher than that in the blank control group and the negative control group, and the difference was statistically significant (p<0.05). *Compared with the miR-7mimics group (p<0.05).

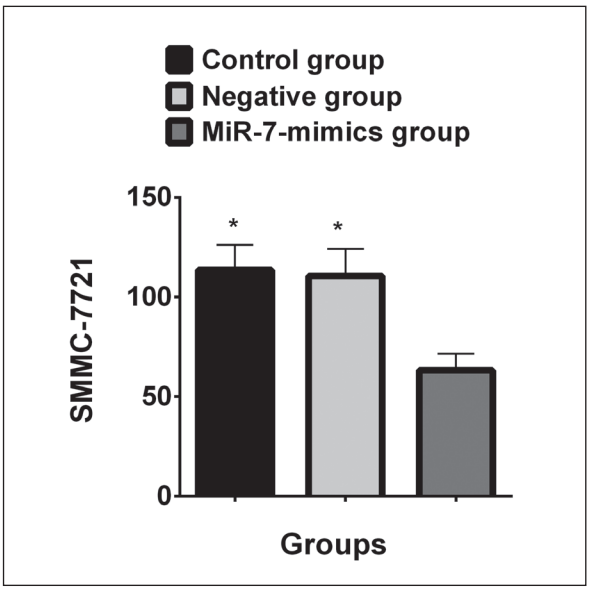

Figure 3. Effect of miRNA-7 on SMMC-7721 invasion. The number of invasion in the miR-7mimics group was significantly lower than that in the negative control group and the blank control group, and the difference was statistically significant ($p<0.05$). *Compared with the miR-7mimics group ($p<0.05$).

proliferation, differentiation, invasion, migration and apoptosis could be controlled by regulating the stability of target mRNA or inhibiting its translation. It plays an important role in the development

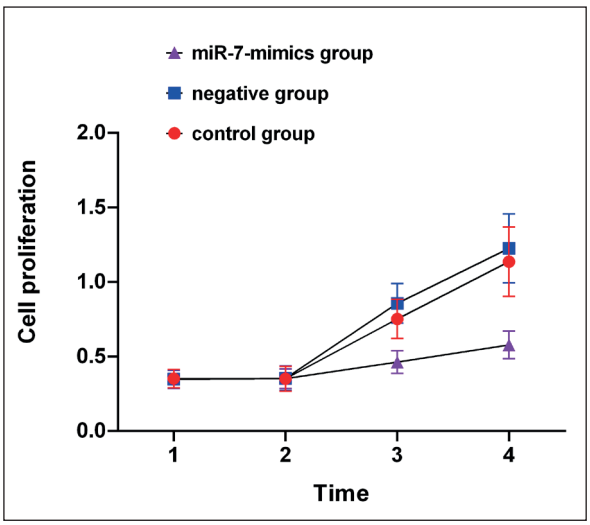

Figure 4. Effect of miRNA-7 on proliferation of SMMC-7721. At 3 d and 4 d, the cell proliferation of miR-7mimics cells was significantly lower than that of the blank control group and the negative control group, and the difference was statistically significant ($p<0.05$). *Compared with the miR-7mimics group at the same time point ($p < 0.05$).

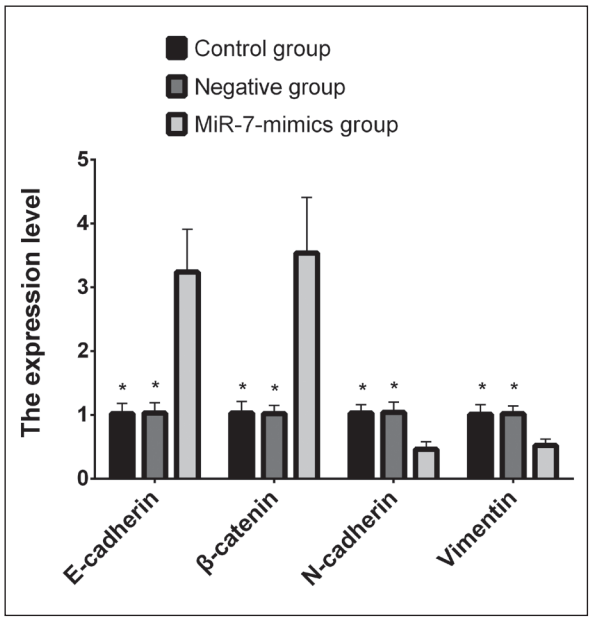

Figure 5. Effect of miRNA-7 on the expression of E-cadherin, β-catenin, N-cadherin and Vimentin. Compared with the blank control group and the negative control group, the expressions of E-cadherin and β-catenin in SMMC-7721 cells transfected with miR-7 in miR-7mimics group were significantly increased ($p<0.05$), while N-cadherin and Vimentin expressions were significantly decreased ($p<0.05$). *Compared with the miR-7mimics group ($p<0.05$).

of tumors[17,18]. MiRNA is also the main regulator of EMT, dynamically regulating the balance between EMT and reverse processes[19,20]. As a member of the miRNA family, Cao et al[21] have shown that miR-7 can inhibit the proliferation, migration and invasion of non-small cell lung cancer cells; but there are still few clinical study on the effect of miR-7 on HCC. Therefore, we took liver cell SMMC-7721 as the object to investigate the effect of miR-7 on the proliferation, invasion and migration ability of SMMC-7721 and EMT.

In our research, 30 patients with HCC admitted to Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology were enrolled in the study group, and 30 patients with concurrent physical examinations were selected as the control group. Strict inclusion and exclusion criteria were implemented. In the study, the eukaryotic expression vector pcDNA3.1(-)-pri-miR-7 (p-miR-7) was used to transiently transfect SMMC-7721 cells *in vitro*, and the expression of microR-7 was detected by RT-qPCR. Western blot was used to detect the expression of EMT marker proteins E-cadherin, β-catenin, N-cadherin and Vimentin. The proliferation of SMMC-7721 cells was detected by CCK-8 assay, and the invasion and migration

ability of cells were detected by transwell assay. Our results showed that the proliferation, invasion and migration of SMMC-7721 cells transfected with miR-7 were significantly reduced, and the expressions of E-cadherin and β-catenin were significantly increased, while the expressions of N-cadherin and Vimentin were significantly decreased.

One study showed that in pancreatic cancer, miR-7 can inhibit autophagy through up-egulated hepatase-AMP-activated protein kinase-activated and mammalian sirolimus target protein (LKB1-AMPK-mTOR) signaling pathway under stressful tumor microenvironment. It also directly targets at autophagy induction and vesicle lengthening stage, reducing the supply of intracellular glucose to glycolysis metabolism. In addition, miR-7 inhibits the proliferation and metastasis of pancreatic cancer cells in vivo and *in vitro*. Lentivirus-mediated miR-7 interferes with glycolysis by inhibiting autophagy, effectively reducing the growth of pation-derived xenografts.

Gu et al[22] showed that miR-7 may act as an important regulatory factor to weaken the glucose pool derived from autophagy, inhibiting the progression of pancreatic cancer. It may be a potential therapeutic target for pancreatic cancer. Our investigation indicated that miRNA-7 can inhibit the proliferation and invasion of HCC cells. We also studied the effect of miR-7 on EMT. In another work, miR-7 was significantly downregulated in cervical cancer, especially metastatic tumors. The ectopic expression of miR-7 significantly inhibited the metastasis and invasion of human cervical cancer cells and C33A cells, and the upregulation of miR-7 significantly inhibited focal adhesion kinase (FAK), which was negatively correlated with miR-7 in cervical cancer tissues[23]. In the two reports, the mechanism of action was found. The specific mechanisms of action of miR-7 in different cancers are different, and the target is different. However, our literature does not specifically address this point. We believe that its mechanism may be related to upregulation of E-cadherin and β-cadherin, and downregulation of N-cadherin and Vimentin proteins, and we hope to conduct this discussion in future studies.

Studies have pointed out that the role of miRNA-7 is not limited to cancer. It expands in health, focusing on organ differentiation and development, as well as in various mammalian diseases, especially in the brain, heart, endocrine pancreas and skin. It indicates that the more we know about miR-7, the more we can recognize the complexity of its regulation and its potential functional applications from biomarkers and therapeutic perspectives[24]. Pollock et al[25] showed that miR-7 prevents progenitor cell apoptosis by regulating the target proteins Ak1, p21, and other possible targets in the p53 pathway, and allows subsequent generation of neurons to control normal brain size. In another study[26], miRNAs have been shown to be useful biomarkers for diagnosing diseases and different types of cancer. Therefore, not only miR-7 can be applied to treatments, but also the miRNA family. They can be applied in cancer as well as other disease-related diagnosis and health-related regulation. We boldly speculate that miRNA will be a huge success in future clinical research and applications.

The present investigation showed the role of miR-7 in the SMMC-7721 cell line and its effect on its EMT, but there are still some limitations. Our research did not further investigate the ability of miR-7 to invade and apoptosis in SMMC-7721 cell line. In addition, we speculate that the mechanism may be related to upregulation of E-cadherin, β-catenin protein and downregulation of N-cadherin and Vimentin proteins, however, we have not validated targeted genes and factors. The specific mechanism is not clear, so we hope to further supplement it in future research.

Conclusions

In brief, miR-7 has an inhibitory effect on the proliferation and invasion of human hepatocellular carcinoma cell line SMMC-7721, which may be related to upregulation of E-cadherin, β-catenin protein and down-regulation of N-cadherin and Vimentin proteins.

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Conflict of Interests

The authors declare that they have no conflict of interest .

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