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MiR-199 inhibits EMT and invasion of hepatoma cells through inhibition of Snail expression

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Abstract. – OBJECTIVE: MiR-199 expression is associated with liver cancer. Bioinformatics analysis revealed that miR-199 has a complementary binding site to the 3'-UTR region of Snail mRNA. This study investigated whether miR-199 plays a role in regulating Snail expression and affecting epithelial-mesenchymal transition (EMT) and invasion of hepatoma cells.

PATIENTS AND METHODS: The Dual-Luciferase reporter gene assay validated the targeted regulation between miR-199 and Snail. QRT-PCR was used to detect and compare the expression of miR-199 and Snail mRNA in human normal liver HL7702 cells, low metastatic MHCC97L cells and high metastatic MHCC97H cells. MHCC97H cells were cultured *in vitro* and divided into two groups: miR-NC group and the miR-199 mimic group followed by the analysis of the expression of Snail, E-cadherin and N-cadherin, as well as cell invasion ability by transwell assay.

RESULTS: There was a targeted regulatory relationship between miR-199 and Snail mRNA. Compared with HL7702 cells, miR-199 expression was significantly decreased, and Snail expression was significantly increased in MHCC97L and MHCC97H cells, with more changes being observed in high metastatic MHCC97H cells. The transfection of miR-199 mimic significantly downregulated the expression of Snail and N-cadherin in MHCC97H cells, increased E-cadherin expression, inhibited the cell's EMT process, and invasion.

CONCLUSIONS: The decrease of miR-199 expression plays a role in upregulating the expression of Snail and promoting EMT and invasion of hepatocarcinoma cells. The increase of the expression of miR-199 can inhibit the expression of Snail and inhibit the EMT process and invasion ability of hepatoma cells.

Key Words:
MiR-199, Snail, Liver cancer, EMT, Invasion.

Introduction

Hepatocellular carcinoma (HCC) is a clinically common malignant tumor. Its morbidity and mortality rank among the top in global malignant tumors. The disease progresses rapidly; the treatment effect is poor with a high mortality rate, which causes serious threats to patients and brings huge burdens to families[1].

Epithelial-mesenchymal transition (EMT) of tumor cells is closely related to tumor invasion, distant metastasis, and postoperative recurrence[2-4]. The zinc finger protein transcription factor Snail, also known as Snai1, downregulates the expression of E-cadherin during EMT and upregulates the expression of N-cadhetin, thereby promoting the EMT process of tumor cells and enhancing their invasive ability[5,6]. Multiple studies[7-9] have shown that abnormal expression of Snail is associated with increased EMT, invasion, and metastasis in multiple tumors. In addition, studies[10-12] have shown that the abnormal expression of Snail is involved in the regulation of EMT, invasion, and metastasis of hepatoma cells.

MicroRNA is an endogenous non-coding single-stranded small molecule RNA of about 22-25 nucleotides in eukaryotes. By complementary binding to the 3'-untranslated region of the target gene mRNA (3'-untranslated region, 3'-UTR), microRNA regulates the expression of target genes by degrading mRNA or inhibiting mRNA translation, and thus participates in the regulation of biological processes such as cell proliferation, differentiation, and migration, and is closely related to tumor occurrence, progression, and metastasis. Scholars[13-15] have shown that the abnormal expression of miR-199 is closely related

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to the occurrence and progression of liver cancer. Bioinformatics analysis showed that there is a targeted complementation relationship between miR-199 and Snail's 3'-UTR. This study investigated whether miR-199 plays a role in regulating Snail expression and affecting EMT processes and invasion of hepatoma cells.

Materials and Methods

Main Reagents and Materials

Human high metastatic MHCC97H liver cancer cells and human low metastatic MHCC97L liver cancer cells were purchased from Nanjing Kezhen Biotechnology Co., Ltd. (Nanjing, Jiangsu, China); human normal liver HL7702 cells were purchased from Shanghai Gaining organism (Shanghai, China); HEK293T cells were purchased from Wuhan Punosi organism (Wuhan, China); DMEM medium, fetal bovine serum (FBS), and penicillin were purchased from Gibco (Grand Island, NY, USA); Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA); RNA extraction reagent Easy RNA Kit and fluorescent quantitative PCR agent TransScript Green One-Step qRT-PCR SuperMix were purchased from Beijing Quan Biological (Beijing, China); miR and miR-199 mimic were designed and synthesized by Guangzhou Ruibo Bio (Guangzhou, China); rabbit anti-E-cadherin, N-cadherin polyclonal primary antibody, HRP-conjugated secondary antibody were purchased from American Abcam (Cambridge, MA, USA), rabbit anti-human Snail and β-actin polyclonal antibody were purchased from Cell Signaling Technology (Danvers, MA, USA); transwell was purchased from Millipore (Billerica, MA, USA); Matrigel was purchased from BD Biosciences (San Jose, CA, USA); Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA); pMIR plasmid was purchased from Changsha Yubao (Changsha, Hunan, China); TGF-β1 recombinant protein was purchased from American R&D Systems (Minneapolis, MN, USA).

Cell Culture and EMT Induction

HL7702, MHCC97H, and MHCC97L cells were cultured in DMEM medium containing 10% FBS, in a cell culture incubator containing 5% CO_2 at 37°C, and sub-cultured at a ratio of 1:4. The cells in the logarithmic phase were used for experiments.

In the EMT induction experiment, MHCC97H cells were seeded in 6-well plates at a density of 3×10^4. After adherence for 24 h, TGF-β1 was added to the medium to a final concentration of 20 ng/mL, treating cells for 96 h to induce MHCC97H cells. In the EMT process, a group which TGF-β1 was not added was used as control.

Dual-Luciferase Gene Reporter Assay

Using the MHCC97H cell genome as a template, the full-length 3'-UTR fragment of Snail gene was amplified, and the PCR product was digested. The amplified product was ligated into pMIR plasmid and transformed to DH5α competent cells. The positive clones were screened, and the corresponding plasmids were picked for transfection and subsequent experiments and designated as pMIR-Snail-WT and pMIR-Snail-MUT respectively.

pMIR-Snail-WT (or pMIR-Snail-MUT) was transfected into HEK293T cells with miR-199 mimic (or miR-NC) using Lipofectamine 2000. After 48 h of culture, the relative Luciferase activity was detected to follow the instructions of the Dual-Glo Luciferase Assay System Kit.

Transfection and Grouping

MHCC97H cells were cultured *in vitro* and divided into two groups: miR-NC transfection group and miR-199 mimic transfection group. The general procedure for transfection was: dilute 10 μL Lip 2000, 50 nmol miR-NC, 50 with 100 μL Opti-MEM. Nmol miR-199 mimic was incubated for 5 min at room temperature, a mix of Opti-MEM with Lipofectamine 2000, miR-NC or miR-199 mimic were incubated for 20 min at room temperature, we added the transfectant mixture to the cell culture medium, we continued to culture 72 hours, and then, we collected the cells for testing.

qRT-PCR Detection of Gene Expression

One-step qRT-PCR was used to detect the relative expression of genes using TransScript Green One-Step qRT-PCR SuperMix in the 20 μL reaction system including: 1 μg of RNA template, 0.3 μM of pre-primer, 0.3 μM of post-primer, 10 μL of 2 × TransStart Tip Green qPCR SuperMix, 0.4 μL of RT Enzyme Mix, 0.4 μL of Dye II, and deionized water. qRT-PCR reaction conditions were: 45°C, 5 min, reverse transcription; 94°C, 30 s; (94°C, 5 s; 60°C, 30 s) × 40 cycles, detection of gene expression on Bio-Rad CFX96 Real Time-PCR in-

strument. The primer sequences for miR-199 was: F-5'-AGAAGGCGATTGATACGAGTCA-3' (sense) and 5'-GGTCTCCCCAGTGTTCAGATA-3' (antisense); U6: 5'-GTGCAGGGTCCGAGGT-3' (sense) and 5'-CGCTTCGGCAGCACAT-3' (antisense); Snail: 5'-TTCTTCTGCGCTACTGCTGCG-3' (sense) and 3'-GGGCAGGTATGGAGAGGAAGA-5' (antisense); GAPDH: 5'-TGGTATCGTGGAAGGACTCATGAC-3' (sense) and 3'-ATGCCAGTGAGCTTCCCGTTCAGC-5' (antisense); E-cadherin: 5'-TCCCATCAGCTGCCCAGAAA-3' (sense) and 3'-TGACTCCTGTGTTCCTGTTA-5' (antisense); N-cadherin: 5'-AGGGTGGACGTCATTGTAGC-3' (sense) and 5'-CTGTTGGGGTCTGTCAGGAT-3' (antisense).

Western Blot

The total protein was extracted from RIPA lysate. After quantification of the protein concentration by BCA method, 40 μg protein was separated on 12% SDS-PAGE, transferred to PVDF membrane (250 mA, 100 min), blocked with 5% skim milk powder at room temperature for 60 min, and incubated with the primary antibody at 4°C overnight (E-cadherin, N-cadherin, S β-actin dilution ratios were 1:2000, 1:2000, 1:5000). After washing the membrane 3 ti with PBST, HRP-conjugated secondary antibo (1:8000 dilution) was added and i ted for 6 min at room temperature follo by hing the membrane 3 times with PB adding hanced chemiluminescence (ECL ution in exposing and developi und rK.

Transwell Ass nalysis of Invasion

100 μL of M l was place the upper surface of the tran l chamber filter. After gel poly zation, 500 complete medium conta 10% FBS was d to the 24-well plat nd the answell chamber containing Matr el w placed in a 24-well plate. 200 μL of M H cells suspended in serum-free DMEM dium then added to the upper mber, to culture for 48 h, discarded medium e transwell upper chamber, and the cells that failed to pass through the on swab. After methanol fixation and crystal violet staining, cell invasion was observed nder an inverted microscope.

Ethics Statement

All research subjects signed the informed consents. This study has been approved by the Ethical Committee of Jining No. 1 People's Hospital.

Statistical Analysis

Statistical analysis was performed using the Statistical Product and Service Solution (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were expressed as m standard deviation (SD). The Student's *t*- was used to compare the measurement da etween groups. $p<0.05$ was considered statisti signi-ficant.

Res

A Targeted Reg ion R onshi Between MiR- and Sna

Bioinforma alysis revea d com-plementary ding between miR-199 and the 3'-UTR of Snail A (Figure 1A). The Dual- ase gene rep assay showed that the nsfection of miR-199 mimic significantly re ed the relative Luciferase activity in pMIR-S -WT transfe d HEK293T cells, while the tra ction of mi NC or miR-199 mimic did not have ficant ect on relative Luciferase activity in p ail-MUT-transfected HEK293T lls (Figure 1B), indicating a targeted regulatory hip between miR-199 and Snail mRNA.

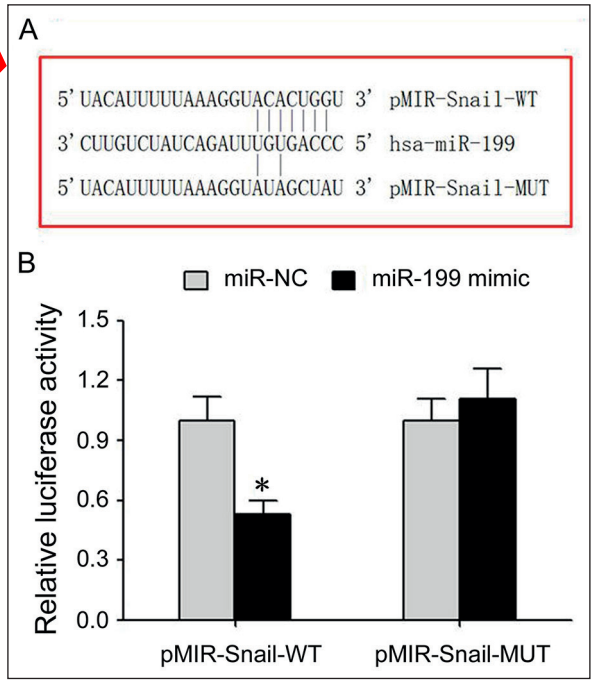

Figure 1. There is a targeted regulation relationship between miR-199 and Snail. **A,** Schematic diagram of a targeted binding site between miR-199 and Snail. **B,** Dual-Luciferase reporter gene assay. *Represents $p<0.05$ compared to miR-NC.

Abnormal Expression of MiR-199 and Snail in Liver Cancer Cells

The results of qRT-PCR showed that, compared with human normal liver HL7702 cells, the expression of miR-199 in liver cancer cells MHCC97L and MHCC97H was significantly decreased, while the expression of Snail mRNA was significantly increased with more changes being observed in highly metastatic MHCC97H cells (Figure 2A). Western blot analysis showed that compared with HL7702 cells, the expression of Snail protein in MHCC97L and MHCC97H cells was significantly increased, and the expression of Snail protein in MHCC97H cells was significantly higher than that in MHCC97L cells (Figure 2B).

Transfection of MiR-199 Mimic Significantly Inhibits EMT Process

The results of qRT-PCR showed that the expression of E-cadherin mRNA in MHCC97H cells was significantly downregulated and the expression of N-cadherin mRNA was upregulated during the EMT of MHCC97H cells induced by TGF-β1 treatment (Figure 3A). The resu qRT-PCR showed that the expression of mi (Figure 3B) and E-cadherin mRNA (Figure was significantly increased in MHCC97H c of miR-199 mimic transfection comp red with a miR-NC group (F and th expression of N-cadherin NA (ure 3C) was significantly reduced tern showed that compare with

sfection of miR-199 mimic significantly reduced the expression of Snail and N-cadherin protein in MHCC97H cells, and significantly increased the expression of E-cadherin protein (Figure 3D).

Transfection of MiR-199 Mimic Significantly Attenuated the Invasion of MHCC97H Cells

The results of the transwell as y sho hat the invasive ability of MHC H cells i miR-199 mimic transfecti roup w s sign cantly reduced, and the n er of i sions was significantly reduced comp the miR IC group (Figure 4).

D ssion

Th nce of HCC fifth in malignant tu , and the disease pro esses rapidly, with hi malignancy, easy invasion and metastasis, hi postoperati recurrence rate, poor survival and prognos [8]. Therefore, studies on the path sis of C and abnormal signal transduction es are important for improving diagnosis, treatment, and prognosis.

is the initial step in the acquisition of mo- cell ability to invade and metastasize, and is closely related to the surrounding tissue invasion, distant metastasis, postoperative recurrence, and poor prognosis[2-4]. Snail is an important regulator of EMT and can downregulate the expression of E-cadherin, a negative regulator of EMT, by bin-

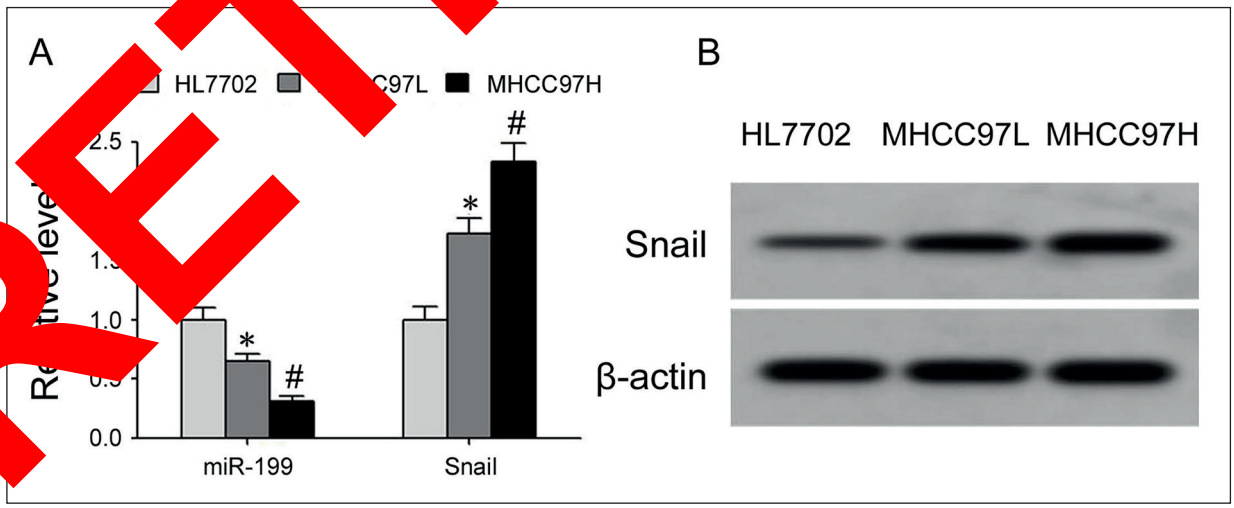

Figure 2. Abnormal expression of miR-199 and Snail in liver cancer cells. **A,** qRT-PCR was used to detect the expression of miR-199 and Snail mRNA in cells. **B,** Western blot analysis of intracellular Snail protein expression. * represents p<0.05 compared with HL7702 cells; # represents $p<0.05$ compared with MHCC97L cells.

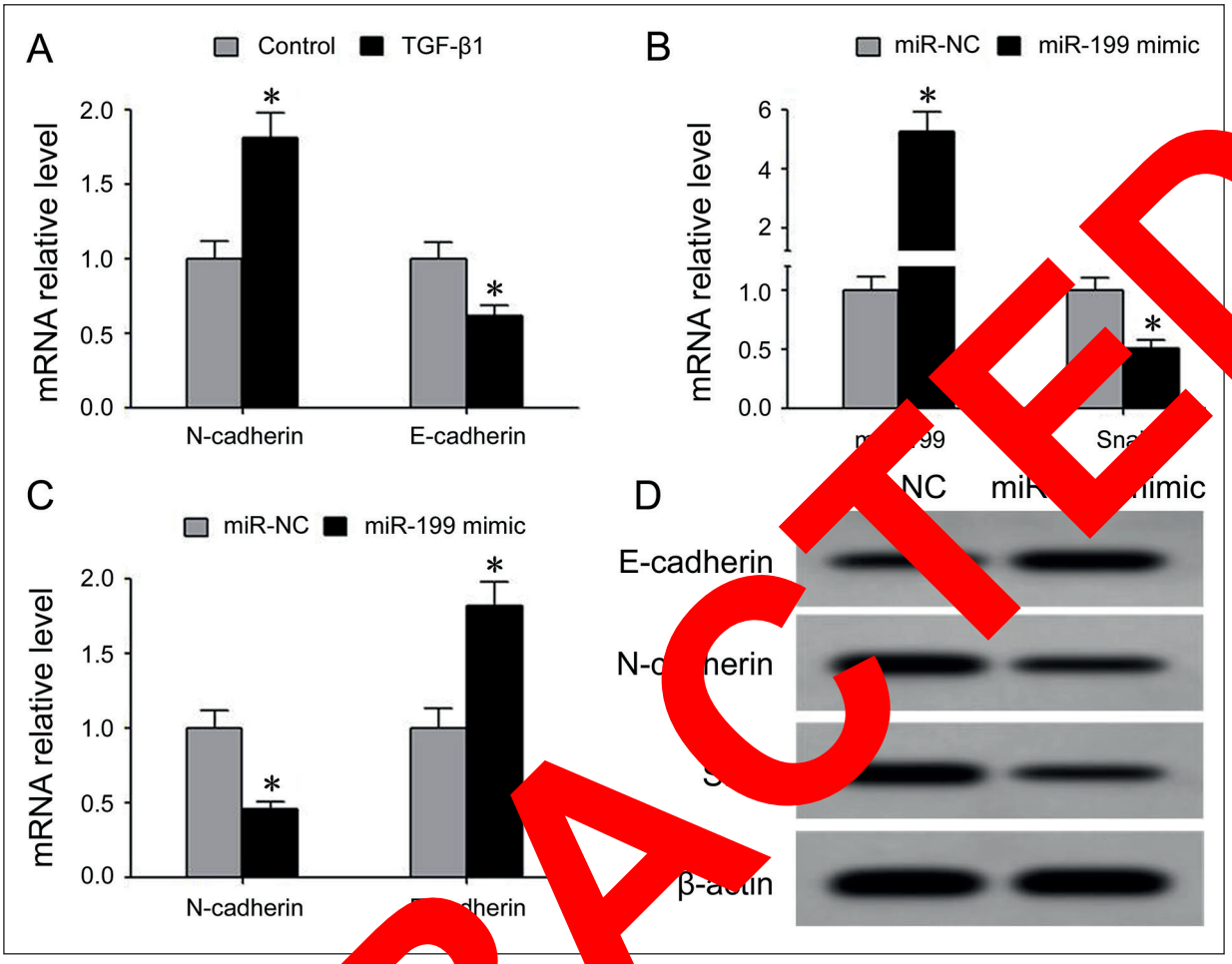

Figure 3. Transfection of miR-199 mimic significantly inhibited EMT process in MHCC97H cells. (**A**) qRT-PCR was used to detect the expression of N-cadherin and E-cadherin mRNA in cells; (**B**) qRT-PCR was used to detect the expression of miR-199 and Snail mRNA in cells; (**C**) qRT-PCR was used to detect the expression of N-cadherin and E-cadherin mRNA in cells; (**D**) Western blot detection of intracellular protein expression. * represents $p<0.05$ compared to the two groups.

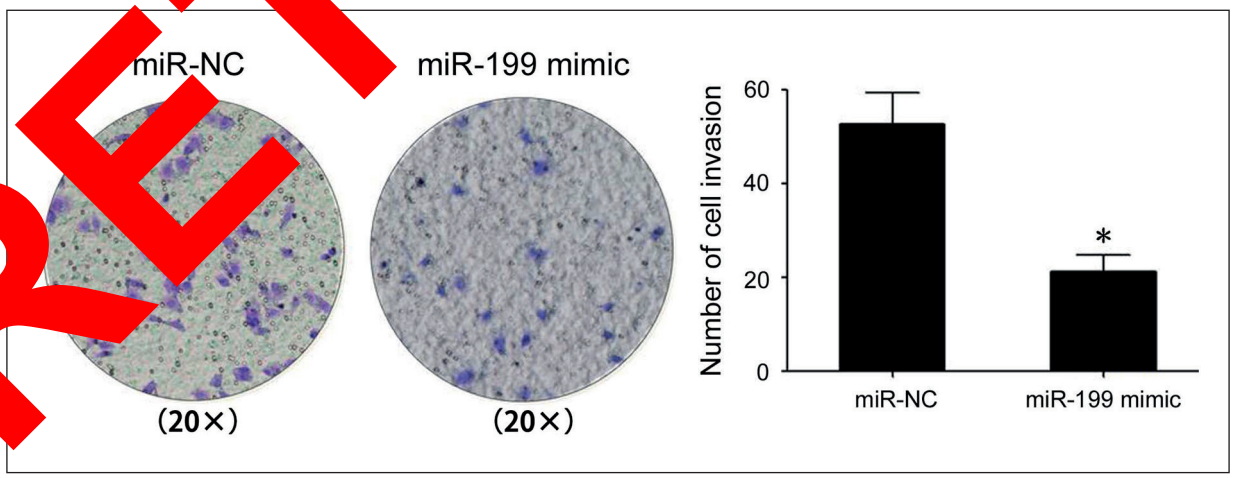

Figure 4. Transfection of miR-199 mimic significantly attenuated the invasion of MHCC97H cells. * represents $p<0.05$ compared to the miR-NC group.

ding to the E-box region of E-cadherin gene promoter. The adhesion is reduced, the cell junction becomes loose, and the expression of the EMT positive regulator N-cadherin is upregulated, thereby promoting the EMT process of tumor cells and enhancing their invasive ability[5,6]. A number of studies have shown that abnormal expression of Snail is closely related to EMT, invasion, and metastasis of various tumors such as breast cancer[7], lung cancer[8], and intestinal cancer[9]. A number of studies have shown that miR-199 is associated with the occurrence, progression, metastasis of several tumors such as lung cancer[19], intestinal cancer[20], and bladder cancer[21]. Studies[13-15] have shown that the abnormal expression of miR-199 is closely related to the occurrence and progression of liver cancer. This study investigated whether miR-199 plays a role in regulating the expression of Snail and affecting the process and invasion of hepatoma cells.

In this research, the Dual-Luciferase gene reporter assay showed that the transfection of miR-199 mimic significantly reduced the relative Luciferase activity in pMIR-Snail-WT transfected HEK293T cells, but miR-199 mimic did not an effect on the relative Luciferase activi the HEK293T cells transfected with pMIR-S MUT, confirming the targeted regulation bet en miR-199 and Snail. These show that, compared with human n HL7702 cells, the expression of miR was si ficantly decreased, while the exp n of S si- gnificantly increased i liver ells C97L and MHCC9 with mo anges found in high metastati CC97H cel e findings showed that th e of miR-19 pression may play a ro in up ting the Snail expression and moting the r tion of liver cancer patho sis and metastasi the study of the rela ship b een miR-19 and liver cancer, L al[22] ved that the expression of miR-199 in t sues of C patients was signifi- antly ased pared with adjacent tissues, l the e of its target genes XBP1 and lin D w significantly increased. Zhan et observed that the expression of miR-199 was y decreased in HCC tumor tissues and cells compared with adjacent tissues. Our study also found that the decrease in miR-199 expres- n was associated with poor prognosis. Zhang et al[13] detected that the expression of miR-199 was abnormally decreased in the liver cancer cell line. Amr et al[23] indicated that compared with patients with chronic hepatitis, the expression of miR-199 in peripheral blood of patients with HCC was significantly decreased; and the expression of miR-199 in HCC tumor tissues was decreased compared with adjacent tissues. ROC curve analysis showed that the decrease of the ex sion of miR-199 has a high diagnostic for HCC. Giovannini et al[14] found that the ression of miR-199 in tumor tissues of HCC p ts was abnormally decreased, and there s a sig ant negative correlation with the t et gene N In the present study, we d onstrat that expression of miR-199 i ated t er cancer, which was consistent with Zhan et al[15], Zhang et al[13], Amr [23].

EMT refers t biological es transforming epith ls into mese al cells. The decrea of E- erin expression, which mediates tight junctio tween cells and cells, is an ant marker MT process. EMT pro ses are closely relate to tumor cells progr , metastasis, recurrence, and poor prognosi [5]. In this re , MHCC97H cells were treate ith TGF-β induce EMT in MHCC97H cells MH 97H cells were transfected with mi miR-199 mimic to observe the anges in cellular EMT process. The results that compared with miR-NC, the tran- ecti of miR-199 mimic significantly reduced the expression of Snail and N-cadherin, upregulated the expression of E-cadherin, inhibited the cell EMT process, and cell invasion. In the study of the relationship between miR-199 and the biological effects of liver cancer cells, Lou et al[22] revealed that there is a mutual regulation between miR-199 and the oncogene XBP1 and cyclin D in Hep3B2.1-7 cells. Overexpression of miR-199 inhibits the proliferative activity of Hep3B2.1-7 cells by inhibition of XBP1 and cyclin D expression. Zhan et al[15] verified that increasing the expression of miR-199 in hepatocellular carcinoma cells can significantly inhibit cell proliferation and attenuate the migration and invasion of hepatoma cells, and the anti-cancer effect is achieved by inhibition of ROCK1. Zhang et al[13] showed that the overexpression of miR-199 in hepatocarcinoma cells can inhibit the proliferation of hepatocarcinoma cells and attenuate cell migration and invasion by significantly inhibiting the expression of RGS17 gene, while the overexpression of RGS17 can antagonize the anti-cancer effect of miR-199. This study combines the targeted regulatory relationship between miR-199 and Snail, revealing that the decreased expression of miR-199

plays a role in upregulating Snail expression and promoting EMT and invasion in hepatoma cells, while increasing miR-199 expression can inhibit the EMT process and invasion of liver cancer cells by targeting inhibition of Snail expression, which has not been reported previously. However, whether the regulatory relationship between miR-199 and Snail and its effect on invasion of liver cancer plays a role in the human body remains unclear and requires further research.

Conclusions

The decrease of miR-199 expression plays a role in upregulating the expression of Snail and promoting EMT and invasion of hepatocarcinoma cells. Increasing the expression of miR-199 can inhibit the expression of Snail and repress the EMT process and invasion ability of hepatoma cells.

Conflict of Interest

The Authors declare that they have no conflict of int

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