miR-185 inhibits cell migration and invasion of hepatocellular carcinoma through CDC42

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Abstract. Hepatocellular carcinoma (HCC) is a primary liver cancer with high incidence and mortality. miR-185, a microRNA with approximately 22-28 nucleotides, was reported to be involved in many cancers. The potential mechanism of miR-185 on HCC through cell division cycle 42 (CDC42) was investigated. RT-qPCR was used to measure the RNA level of miR-185 and CDC42 in HCC tissues and cells. The dual luciferase reporter assay was used to verify whether CDC42 was a target gene for miR-185. Transwell assay was employed to detect the ability of migration and invasion to change miR-185. miR-185 expression was low in HCC and negatively correlated with CDC42. miR-185 inhibited HCC migration, invasion and miR-185 low expression predicted poor prognosis. CDC42 was predicted to be a target gene for miR-185, and regulated by miR-185. miR-185 suppressed the ability of cell migration and invasion through CDC42 in HCC. It is suggested that miR-185/CDC42 axis may present a novel target for HCC treatment.

Introduction

Primary liver cancer is a malignant tumor, incidence rate was the fifth and the mortality ranked third in 2007, and approximately 85-90% was hepatocellular carcinoma (HCC). Sub-Saharan Africa and Eastern Asia are the two most common places for HCC, of which the incidence in China was more than half of the whole world (1). Metastasis and recurrence often occur in HCC due to factors, such as geographical location, race, sex, environment, and molecular factors. Therefore, identification of tumor biomarkers for early diagnosis is essential for HCC patients.

Materials and methods

Tissue samples and cell lines. Paired tumors and non-carcinomatous tissue were available from 63 patients with surgery from Central Hospital of Zibo. All the specimens were obtained with informed consent of the patients and the study was approved by the Ethics Committee of Central Hospital of Zibo (Zibo, China).

HuH-7 human hepatocellular carcinoma cell and the human normal hepatocyte L-02 cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA,
Cells were incubated at constant temperature at 37°C with 5% CO₂.

Western blotting. For western blotting, the first cells were lysed using RIPA lysis buffer containing PMSF (both from Beyotime, Shanghai, China) on ice. Following centrifugation with 12,000 x g at 4°C, the concentration of protein was measured by BCA reagent kit (Solarbio, Beijing, China) and the absorbance was measured by a microplate reader. SDS-PAGE was applied for separation and transfer onto a PVDF membrane, and it was incubated at 4°C overnight with mouse anti-CDC42 monoclonal antibody (cat. no. ab41429; 1:1,000; Abcam, Cambridge, UK). After washing with TBST buffer (Tris-buffered saline with Tween-20, pH 8.0), the blots were incubated with anti-mouse IgG (1:3,000; cat. no. SAB4600004 Novus Biologicals, Littleton, CO, USA) at room temperature for 2 h. The protein signal was detected with chemiluminescence using the Bio-Rad Gel Doc XR instrument (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

Transwell assay. Transwell assay was used to test the ability of migration and invasion. The Transwell chambers (Costar, Corning, NY, USA), were 8 µm in size, with or without Matrigel (Clontech, Mountain View, CA, USA). The chambers within 200 µl cell suspension was put into 24-well plate containing 500 µl medium. Culturing at 37°C with 5% CO₂ for about 48 h before the cells moved under the chambers. After fixed with methanol for 20 min, the cells were stained using crystal violet and observed by microscope (BX51 Olympus, Shenzhen, China).

RT-qPCR. Total RNAs were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) and miRNAs using miRcute Extraction and Separation of miRNAs kit (Tiangen, Beijing, China). Reverse transcription of purified RNAs was used PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) conducted by two steps at 37°C for 5 min and 42°C for 25 min in 20 µl reaction system. STEP ONE RT-qPCR Apparatus (Applied Biosystems, Foster City, CA, USA) and miRNA SYBR Green RT-qPCR kit (ABM, Inc., USA) were employed to perform the quantitative real time PCR. The following thermocycling conditions were used for PCR: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. GAPDH and U6 were used as normalization for CDC42 and miR-185, respectively. All the primers were purchased from Genechem (Shanghai, China), which were CDC42 forward: 5'-GCTCTAGAGCCCTTAAGGGGAG GAG-3' and reverse: 5'-GCTCTAGAAAAATCTCTTATCAA CAC-3'; U6 forward: 5'-CTCAGTTCCGACGACA-3' and reverse: 5'-AACGTTTCCAAGGCTGTGCTT-3'; miR-185 forward: 5'-CAATTGGAGAGAAAGGATGCAG-3' and reverse: 5'-AATCCATGAGAGATCCCTACCG-3'; GAPDH forward: 5'-GGTGAGAATTGCGGAGTCAACG-3' and reverse: 5'-CAAGGGTGTCCAGGATGACC-3'.

Plasmid construction and luciferase reporter assay. TargetScan (www.targetscan.org), online software, predicted CDC42 was a target gene of miR-185 with binding site at 647-654 of its 3'UTR. In order to verify whether miR-185 interacts with CDC42, double luciferase reporter assay was performed. The 3'UTR oligonucleotide fragment of CDC42 was inserted into pcDNA3.1 plasmid vector (pcDNA3.1-CDC42-WT). Mutated the binding site from 5'-...UCUCUC...-3' to 5'-...AGAGAGG...-3' and then inserted into pcDNA3.1 plasmid vector (pcDNA3.1-CDC42-MUT). The effectiveness of cloning was detected by sequencing.

HuH-7 cells at 80% confluence were seeded in 6-well plates and cultivated overnight before transfection. miR-185 mimic or negative control (NC) and pcDNA3.1-CDC42-WT or pcDNA3.1-CDC42-MUT were co-transfected into HCC HuH-7 cells using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Then the transfected cells were cultured at 37°C with 5% CO₂ for 48 h and harvested for analysis.

Transfection. The influence of miR-185 to cell migration and invasion, was investigated with miR-185 mimic and inhibitor were used to overexpress or knock down miR-185. Similarly, we used small interfering RNA (siRNA) to interfere with the expression of CDC42, and detected the effect of miR-185 through CDC42 for cell migration and invasion.

HCC cells were seeded into a 6-well plate and cultivated overnight to make sure that cells adhered to the wall. The medium was replaced before transfection, and the specific plasmids were transfected in HCC cells by Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

Statistical analysis. Experimental results were demonstrated using SPSS 20.0 software package (IBM Corp., Armonk, NY, USA). The differences between the groups were calculated using t-test or Dunnett's, Fisher's after one-way ANOVA test. In addition, χ² test was used to compare the expression of miR-185 and the clinicopathological features of HCC patients. The Kaplan-Meier method with log-rank test was used to calculate the survival rates. A total of 63 cases of patients were divided into two groups [miR-185(+) and miR-185(−)] according to the miR-185 expression. The effects of the miR-185 expression on overall survival (OS) and disease-free survival (DFS) (in months) were analyzed using the multivariate Cox regression method. For all tests, P<0.05 was considered to indicate a statistically significant difference.

Results

miR-185 expression is significantly low and correlates with CDC42 in HCC. miR-185 and CDC42 expression level of the 63 paired HCC and non-carcinomatous tissues was detected by RT-qPCR. For HCC tissues, the expression of miR-185 was reduced significantly compared with non-carcinomatous tissues (P<0.0001) (Fig. 1A). Similarly, miR-185 expression in HCC cells HuH-7 was decreased in contrast to normal HCC L-02 cells (P<0.0001), as shown in Fig. 1B. On the contrary, CDC42 was overexpressed in HCC cell lines HuH-7 vs. normal liver L-02 cells (P<0.0001) (Fig. 1C). miR-185 was correlated with CDC42 in HCC (r=-0.8049, P<0.0001) (Fig. 1D).

miR-185 inhibits migration and invasion and miR-185 deficiency predicts poor prognosis of HCC patients. In order to test whether miR-185 influenced the progress of HCC, we used mimic or inhibitor to overexpress or knock down the expression of miR-185 in HuH-7 cells. The overexpression (P-value
was <0.0001) or knockdown (P-value was <0.0001) effects are shown in Fig. 2A.

Transwell assay was used to detect the influence of miR-185 on migration and invasion. As shown in Fig. 2B, in knockdown

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Figure 1. miR-185 expression is significantly low and correlates with CDC42 in HCC. (A) The relative expression level of miR-185 in HCC tissues and corresponding non-tumorous tissues. (B) miR-185 expression level in HCC cell lines HuH-7 and normal human HCC L-02 by RT-qPCR. (C) CDC42 mRNA level in HuH-7 was upregulated compared with CDC42 cells. (D) miR-185 expression is associated with CDC42 in HCC. ***P<0.001. CDC42, cell division cycle 42; HCC, hepatocellular carcinoma.

Figure 2. miR-185 inhibits migration and invasion and miR-185 deficiency predicted poor prognosis of HCC patients. (A) The effectiveness of the overexpressed and knockdown miR-185 in liver cancer cells HuH-7. (B) Transwell assay was used to analyze migration and invasion of HuH-7 cells. (C) The OS and DFS of patients with high (n=22) or low (n=41) miR-185 expression. "P<0.01; """P<0.001. HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease free survival.
of miR-185, the cells number that moved to the lower chamber was increased in HuH-7 (P-values for migration and invasion are 0.0029 and 0.0011, respectively) cells, which illustrated the ability of migration and invasion to increase. On the contrary, in the overexpression of miR-185 inhibitor, the cell number was reduced in HuH-7 (P=0.0033 and 0.0042 for migration and invasion) cells.

miR-185 was significantly related to tumor size (P=0.033), TNM stage (P=0.019), lymph node metastasis (P=0.023), as shown in Table I. OS and DFS of patients in miR-185(-) group was lower than that in miR-185(+) group (log-rank P=0.0011 and 0.0116) based on Kaplan-Meier, as shown in Fig. 2C. The results revealed that miR-185 deficiency predicted poor prognosis in HCC.

miR-185 mediates the expression of CDC42 by direct targeting. CDC42 was predicted to be a potential target by online software TargetScan (http://www.targetscan.org/vert_71/), which was reported to be related with tumor progression. The potential binding site of CDC42 for miR-185 is located at 3'UTR. Mutated the binding site from 5'-…UCUCUCC…-3' to 5'-…AGAGAGG…-3', as shown in Fig. 3A. To verify the association between CDC42 and miR-185, we co-transfected miR-185 or negative control and pcDNA3.1-CDC42-WT or pcDNA3.1-CDC42-MUT into HCC cells HuH-7. In co-transfection of miR-185 and pcDNA3.1-CDC42-WT, the relative luciferase activity was reduced compared with co-transfected negative control and pcDNA3.1-CDC42-WT

| miR-185 expression and clinicopathological features in 63 paired HCC. |
|------------------------|------------------------|------------------------|
| Clinicopathological features | Cases (n=63) | miR-185 expression | P-value* |
| Sex | | | |
| Male | 30 | High (11 (36.7)) | 0.782 |
| Female | 33 | Low (9 (33.3)) |
| Age (years) | | | |
| ≤60 | 28 | High (9 (32.1)) | 0.679 |
| >60 | 35 | Low (13 (37.1)) |
| Tumor size (mm) | | | |
| ≤5.0 | 25 | High (13 (52.0)) | 0.029 |
| >5.0 | 38 | Low (9 (28.9)) |
| TNM stage | | | |
| I-II | 26 | High (13 (50.0)) | 0.035 |
| III-IV | 37 | Low (9 (24.3)) |
| Local invasion | | | |
| T1-T2 | 24 | High (12 (48.0)) | 0.077 |
| T3-T4 | 39 | Low (10 (26.3)) |
| Lymph node metastasis | | | |
| Positive | 33 | High (8 (24.2)) | 0.040 |
| Negative | 30 | Low (14 (46.7)) |

*P-values calculated with Chi-square test. HCC, hepatocellular carcinoma.
whereas co-transfected miR-185 and pcDNA3.1-CDC42-MUT had little change compared with NC, and the P-value of HuH-7 was 0.8105 (Fig. 3B). The results show that miR-185 can directly bind to the 3'UTR of CDC42. Previously it was shown that miR-185 can directly bind to the 3'UTR of CDC42, and the expression of miR-185 and CDC42 had a negative correlation. Therefore, we hypothesized that miR-185 could regulate the expression of CDC42. Unfortunately, in overexpressed or knocked down miR-185, the mRNA level of CDC42 had almost no change in either HuH-7 (P=0.074 or 0.8190 for overexpression or knockdown miR-185) cells. On the contrary, the expression in protein level was reduced or increased significantly in HuH-7 cells (Fig. 3C).

**Interference of CDC42 partially blocks the function of miR-185.** To verify whether miR-185 affected cell migration and invasion by regulating CDC42 expression, we carried out a rescue experiment, which directly interfered with the expression of CDC42 to detect the ability of cell migration and invasion. The interference effect was measured by RT-qPCR (P<0.0001 of HuH-7). Whereas, co-transfected miR-185 and pcDNA3.1-CDC42-MUT had little change compared with NC, and the P-value of HuH-7 was 0.8105 (Fig. 3B). The results show that miR-185 can directly bind to the 3'UTR of CDC42. Previously it was shown that miR-185 can directly bind to the 3'UTR of CDC42, and the expression of miR-185 and CDC42 had a negative correlation. Therefore, we hypothesized that miR-185 could regulate the expression of CDC42. Unfortunately, in overexpressed or knocked down miR-185, the mRNA level of CDC42 had almost no change in either HuH-7 (P=0.074 or 0.8190 for overexpression or knockdown miR-185) cells. On the contrary, the expression in protein level was reduced or increased significantly in HuH-7 cells (Fig. 3C).

**Discussion**

Primary liver cancer patients especially with HCC are prone to metastasis and recurrence. It has been reported that tumorigenesis was caused by oncogenes or loss of tumor inhibitors, whereas, several of the molecular biological mechanisms remain unknown (21). miRNAs are important epigenetic regulators and usually act as oncogene or tumor suppressor gene in progression of various cancers (22-28). miR-185 induces cell cycle arrest at the G1 phase to suppress proliferation in human non-small cell lung cancer cells (7). The expression of miR-185 in carcinoma was significantly lower than that in normal tissues, including HCC, colon cancer and prostate carcinoma (6,8,11). In this study, we discovered that miR-185 is usually downregulated in HCC and the average expression level of miR-365 in tumor tissues was obviously lower than that in paracancerous tissues.

To investigate the role of miR-185, we overexpressed or knocked down miR-185 to detect the migration and invasion...
miR-185 was expressed at low level in HCC. Moreover, interference of CDC42 inhibited the migration and invasion of HCC cells. Additionally, we found that depleting CDC42 did not alter the CDC42 mRNA level.

Furthermore, in order to confirm the biological role of CDC42 in HCC, recovery experiment was carried out and it was found that interference of CDC42 inhibited the migration and invasion of HCC cells. Additionally, we found that depletion of CDC42 reversed partial the function of miR-185.

In conclusion, our results suggest that miR-185 is usually expressed at low level in HCC. Moreover, interference of miR-185 promotes the ability of migration and invasion of HCC cells by directly targeting CDC42. Thus, our findings displayed that miR-185 may be developed to a potential diagnostic marker of HCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

QZ contributed to the conception of the study and wrote the manuscript. YC contributed significantly to perform the experiment. KL contributed significantly to the analysis of the data and helped in the writing of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Central Hospital of Zibo (Zibo, China) and informed consent of each patient was received.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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