miR-145-5p Acts as a Novel Tumor Suppresser in Hepatocellular Carcinoma Through Targeting RAB18

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Abstract
Micro-RNAs play critical roles in initiation and progression of hepatocellular carcinoma. However, the biological role of microRNA-145-5p in hepatocellular carcinoma and how it works are still not clearly understood. Expression levels of microRNA-145-5p in hepatocellular carcinoma cell lines were examined by reverse transcription quantitative polymerase chain reaction. Cell counting kit-8, wound-healing assay, and flow cytometry assay were conducted to investigate the role of microRNA-145-5p on proliferation, migration, and apoptosis. Luciferase reporter assay and Western blot were performed to investigate the correlation between microRNA-145-5p and RAB18. We found microRNA-145-5p was downregulated in hepatocellular carcinoma cell lines compared to the normal cell line. Overexpression of microRNA-145-5p inhibited the proliferation and migration but promoted apoptosis of hepatocellular carcinoma cells in vitro. RAB18 was validated a target of microRNA-145-5p and ectopic expression of RAB18 can promote the proliferation and migration but inhibit apoptosis of hepatocellular carcinoma cells. These findings indicate that microRNA-145-5p functions as a novel tumor suppressor through targeting RAB18, suggesting that microRNA-145-5p might be a potential new therapeutic molecules for the treatment of hepatocellular carcinoma.

Keywords
miR-145-5p, RAB18, hepatocellular carcinoma, tumor suppressor, cell behaviors

Abbreviations
HCC, hepatocellular carcinoma; HCV, hepatitis C virus; miRNAs, micro-RNA; 3'UTR, 3'-untranslated region; NSCLC, non-small cell lung cancer; miR-145-5p, microRNA-145-5p; miR-NC, negative control miRNA; wt, wild type; mut, mutant; NC-vector, negative control vector; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; CCK-8, cell counting kit-8

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Introduction
Hepatocellular carcinoma (HCC) was reported to rank as the fourth leading cause of cancer-related death worldwide.1 About 841 000 new cases and 782 000 deaths will occur in 2018 according to the latest epidemiology data.1 In Western countries, the incidence was reported to continuously increase due to chronic hepatitis C virus (HCV) infection.2 Most patients with HCC were diagnosed at a late stage and therefore results in a poor overall survival rate.3,4 Therefore, it is important to investigate the molecular mechanism related to HCC progression with the aim to identify novel therapeutic target for HCC.

Micro-RNAs (miRNAs) are a family of small non-coding single-stranded RNAs of 21 to 30 nucleotides in length that can regulate target gene expression at the post-transcriptional and translational levels mainly through 3'-untranslated region (3'-UTR) binding.5 Mounting evidence has suggested that miRNAs participate in both tumor initiation and progression process of human cancers and have the potential to be...
developed as therapeutic targets. However, the correlation between miRNAs and HCC tumorigenesis remains to be elucidated although significant processes have been made.

MicroRNA-145-5p was reported to be dysregulated in several human cancers and functioned as a tumor suppressor to regulate cell proliferation, metastasis, apoptosis, and epithelial–mesenchymal transition. MicroRNA-145-5p was found downregulated in non-small cell lung cancer (NSCLC) and suppresses the epithelial–mesenchymal transition of NSCLC cells through inactivating the c-Jun N-terminal kinase signaling pathway by targeting mitogen-activated protein kinase 1. Moreover, miR-145-5p was revealed to suppress esophageal squamous cell carcinoma migration and invasion through regulating the specificity protein 1 and nuclear factor kB (p65) pathway. However, the expression and biological role of miR-145-5p in HCC remain largely unknown.

RAB18 belongs to the RAS superfamily and plays a key role in carcinogenesis. It has been reported that overexpression of RAB18 promotes proliferation and chemoresistance of gastric cancer through regulation of mitochondrial function. Moreover, RAB18 expression was revealed to be regulated by miRNAs including miR-455-5p and miR-20b/c in human cancers. These results demonstrated that RAB18 functions as an oncogene in cancer progression.

In this study, we found miR-145-5p expression was downregulated in HCC cell lines. Overexpression of miR-145-5p using miR-145-5p mimic inhibited HCC cell proliferation and cell migration but promoted cell apoptosis. We further identified RAB18 as a direct target of miR-145-5p. The findings of this study will provide new insights into the functions of miR-145-5p as well as its molecular mechanisms in HCC.

Materials and Methods

Cell Line and Cell Culture

One normal hepatocyte cell line (L02) and 3 HCC cell lines (including BEL-7402, Hep3B, and Huh7) were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Hep3B cells were incubated in minimum essential medium (Gibco, Thermo Fisher Scientific, Inc, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc) and 1% penicillin/streptomycin (Beyotime, Haimen, Jiangsu, China). Meanwhile, the rest of the cell lines was cultured in Dulbecco modified Eagle’s medium (Gibco, Thermo Fisher Scientific, Inc) and 1% penicillin/streptomycin (Beyotime, Haimen, Jiangsu, China). The membranes were incubated with 5% of CO2.

Cell Transfection

Cells to be transfected were incubated in 6-well plate and transfected with miR-145-5p mimic, negative control miRNA (miR-NC), RAB18 overexpression construct (RAB18 construct), or negative control vector (NC-vector) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc, Waltham, Massachusetts) according to manufacturer’s instruction. After 48 hours of transfection, the cells were harvested for further analyses. Synthetic miRNAs were purchased from Genepharm (Shanghai, China), while the RAB18 construct and NC-vector was purchased from GenScript (Nanjing, China).

RNA Isolation and Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was isolated from the cultured cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) following standard protocol. First-strand complementary DNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted using SYBRVR GreenER qPCR Supermix Universal (Invitrogen) at the ABI 7500 system (Applied Biosystem, Foster City, California). Relative expression levels of miR-145-5p were calculated using 2^(-DDCT) method with U6 snRNA as internal control. The primer sequences used in this study are presented in Table 1.

Protein Isolation and Western Blot

Total protein was isolated from the cultured cells using Radio Immunoprecipitation Assay (RIPA) buffer containing protease inhibitors (Beyotime). Samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, Massachusetts). The membranes were incubated with primary antibodies (Rabbit anti-RAB18: ab105519; Rabbit anti-GAPDH: ab181602; Abcam, Cambridge, Massachusetts) overnight at 4°C after blocking with 5% fat-free milk. Then, the horseradish peroxidase conjugated goat anti-rabbit secondary antibody (ab6721; Abcam) was added to the membranes for 1 hour at room temperature. Subsequently, the band was visualized using BeyoECL Plus (Beyotime) and analyzed using Image J 1.42 software (NIH, Bethesda, Maryland).

Cell Proliferation Analysis

Cell proliferation was determined using cell counting kit-8 (CCK-8; Beyotime). Cells were seeded in 96-well plates at the density of 5 × 10^3 cells/well and incubated overnight at 37°C. CCK-8 reagent of 10 μL was added to each cell at indicated time and incubated at 37°C for additional 1 hour. Absorbance

Table 1. Primer Sequences Used in This Study.

| Genes     | Sequences                          |
|-----------|------------------------------------|
| miR-145-5p| Forward: 5’-GTCCAGTTTTCAGGAATC-3’  |
|           | Reverse: 5’-AGAACAGTATTTCCAGGAAT-3’|
| U6 snRNA  | Forward: 5’-CTCGTTCGCGCAGCACA-3’   |
|           | Reverse: 5’-AACGCTTCAGAATTTGGCT-3’|

Abbreviation: miR-145-5p, microRNA-145-5p.
of each well was measured at 450 nm in a microplate reader (Thermo Fisher Scientific, Inc).

**Cell Apoptosis Analysis**

Cells were stained with FITC-Annexin V and PI using Annexin V-FITC Apoptosis Detection Kit (Beyotime) according to the protocols. BD FACS Calibur flow cytometer (BD Biosciences, San Jose, California) was used to analyze the stained cells. Cell apoptosis percentage was calculated and compared between each group.

**Wound-Healing Analysis**

Cells were seeded into 6-well plates and incubated to reach 100% confluence. After that, the cells were wounded by a 200-μL pipette tip and then washed with phosphate-buffered saline for 3 times. Wound was photographed using an inverted microscope (Olympus, Tokyo, Japan), and wound closure rate was assessed.

**Targets of miR-145-5p Predicted by TargetScan**

TargetScan (http://www.targetscan.org/vert_72/), an online miRNA targets prediction algorithm, was utilized to predict the targets of miR-145-5p. Among the predicted targets, RAB18, an oncogene in several human cancers as reported in previously reports,12-14 was selected for further experiments.

**Luciferase Reporter Assay**

Wild-type (wt) or mutant (mut) of 3'-UTR of RAB18 were inserted into pGL3-Basic vector (Promega, Madison, Wisconsin) and named as wt-RAB18 or mut-RAB18. After 24 hours of incubation, the cells were co-transfected with wt-RAB18 or mut-RAB18 (500 ng) and miR-145-5p mimic or miR-NC (50 nM) using Lipofectamine 2000. Twenty-four hours after transfection, luciferase activity was measured using dual-luciferase reporter assay (Promega) with Renilla luciferase as internal control.

**Statistical Analysis**

Statistical analysis was performed using the SPSS 22.0 (SPSS, Chicago, Illinois) and expressed as the mean (standard deviation). Differences between the 2 groups were analyzed by Student t test, while 1-way analysis of variance post Tukey post hoc test was used when more than 2 groups were compared. P < .05 was regarded as statistical significance.

**Results**

**Expression of miR-145-5p was Downregulated in HCC Cell Lines**

The expression level of miR-145-5p in HCC cell lines (BEL-7402, Hep3B, and Huh7) and normal liver cell line (L02) was analyzed by RT-qPCR. We found miR-145-5p expression was decreased in HCC cell lines investigated compared to L02 cell line (Figure 1). BEL-7402 and Hep3B cell lines were selected for further studies, as it ranks the first and second lowest miR-145-5p expression in the HCC cell lines investigated (Figure 1).

**Overexpression of miR-145-5p Inhibits HCC Cell Proliferation and Migration but Promotes Apoptosis**

We transfected the miR-145-5p mimic and miR-NC into the BEL-7402 and Hep3B cell lines to investigate the role of miR-145-5p in HCC. Analysis of RT-qPCR results confirmed miR-145-5p mimic transfection significantly elevated the expression of miR-145-5p (Figure 2A). The CCK-8 assay revealed that overexpression of miR-145-5p inhibits the proliferation of both BEL-7402 and Hep3B cell lines (Figure 2B). Furthermore, we examined the effect of miR-145-5p on cell migration. As expected, miR-145-5p overexpression by miR-145-5p mimic decreased cell migration compared to miR-NC (Figure 2C). Using flow cytometry analysis, we found miR-145-5p mimic transfection promoted cell apoptosis (Figure 2D). These results revealed that miR-145-5p overexpression inhibits HCC cell proliferation and migration but promote apoptosis.

**RAB18 Was a Direct Target of miR-145-5p**

TargetScan analysis result revealed that 3'-UTR of RAB18 contains a binding site for miR-145-5p (Figure 3A). Luciferase activity reporter assay revealed that miR-145-5p mimic transfection significantly reduced luciferase activity of BEL-7402 and Hep3B cell lines transfected with wt-RAB18 but not mut-RAB18 (Figure 3B). Western blot analysis result demonstrated that miR-145-5p mimic transfection decreased RAB18 expression in BEL-7402 and Hep3B cell lines (Figure 3C). These results demonstrated that RAB18 was a direct target of miR-145-5p.
RAB18 Functions in HCC Cell Behaviors are Opposite to Those of miR-145-5p

Since RAB18 was a target of miR-145-5p, it was investigated whether RAB18 was a mediator for the biological role of miR-145-5p in HCC. The transfection of RAB18 construct significantly upregulated the expression of RAB18 in BEL-7402 and Hep3B cell lines (Figure 4A). Meanwhile, the inhibitory effect of miR-145-5p mimic on RAB18 expression could be reversed by RAB18 construct (Figure 4A). As presented in Figure 4B, overexpression of RAB18 promoted cell proliferation and partially reversed the effect of miR-145-5p mimic. Furthermore, the wound-healing analysis showed cell migration ability could be enhanced by RAB18 construct (Figure 4C). Also, it was found the inhibitory effect of miR-145-5p mimic could be restored by RAB18 construct (Figure 4C). Moreover, we found cell apoptosis rate could be repressed by RAB18 construct transfection (Figure 4D). These data suggested RAB18 was an effector for the inhibitory effects of miR-145-5p in HCC.

Discussions

Micro-RNAs have been demonstrated to function as either tumor suppressor or oncogene in human cancers. Accumulating studies have demonstrated that miR-145-5p played a critical tumor suppressive role in the progression of human cancers. However, it was not determined whether or not miR-145-5p has a role in the progression of HCC. We examined the expression of miR-145-5p in HCC cell lines and a normal liver cell line. Not surprisingly, we found miR-145-5p expression was significantly reduced in HCC cell line compared to the L02 cell line. Furthermore, we examined the effects of miR-145-5p on HCC cell proliferation, migration, and apoptosis using the CCK-8 assay, wound-healing assay, and flow cytometry assay in vitro. We demonstrated that overexpression of miR-145-5p inhibits HCC cell proliferation and migration but promotes cell apoptosis in vitro. These results showed miR-145-5p also functions as a tumor suppressor in HCC, which is inconsistent with the role of miR-145-5p in other cancer types.

It has been widely accepted that miRNAs exert its function through regulating the expression of their target genes. Previous studies have demonstrated that miR-145-5p serves as a tumor suppressor via targeting several genes including mitogen-activated protein kinase 1, specificity protein 1, nuclear factor xB, and so on. It was also reported that miR-145-5p expression could be regulated by long non-coding RNA or circular RNA. In the current study, we newly identified RAB18 as a possible target of miR-145-5p using the online prediction algorithm TargetScan V 7.2. The 3′-UTR of
RAB18 was predicted to contain a binding sequence for miR-145-5p. Luciferase activity reporter assay revealed that the transfection of miR-145-5p mimic in HCC cell lines significantly reduced the luciferase activity in cells transfected with wt-RAB18 but not mut-RAB18. Meanwhile, Western blot analysis revealed that RAB18 expression in HCC cell lines was significantly reduced by miR-145-5p mimic compared to the miR-NC.

Thereafter, we investigated whether RAB18 mediated the inhibitory effects of miR-145-5p in proliferation, migration, and apoptosis of HCC cells using a series of in vitro assays. Our findings exhibited that RAB18 construct transfection significantly upregulated RAB18 expression. In the meantime, we found RAB18 construct transfection significantly promoted HCC cell proliferation and migration but inhibited cell apoptosis. Furthermore, the inhibitory effects of miR-145-5p on HCC cell behaviors could be reversed by RAB18 construct transfection.

In conclusion, we demonstrated the tumor suppressive role of miR-145-5p in HCC. We identified the miR-145-5p/RAB18 axis in HCC, and this axis inhibits HCC cell proliferation and migration but promotes apoptosis. We propose that miR-145-5p overexpression might be a possible therapeutic target for HCC.

Figure 3. RAB18 was a direct target of miR-145-5p. (A) Putative binding site between miR-145-5p and 3'-UTR of RAB18. (B) miR-145-5p mimic transfection inhibits the luciferase activity of HCC cells transfected with wt-RAB18 but not mut-RAB18. (C) RAB18 expression in HCC cells transfected with miR-145-5p mimic or miR-NC. (***P < .001, ns not significant). miR-145-5p indicates microRNA-145-5p; HCC, hepatocellular carcinoma; miR-NC, negative control miRNA; UTR, untranslated region; wt, wild-type; mut, mutant.
Figure 4. RAB18 was an effector for the inhibitory roles of miR-145-5p in HCC. (A) RAB18 expression, (B) cell proliferation, (C) cell migration, scale bar 100 μm, and (D) cell apoptosis in BEL-7402 cell line transfected with RAB18 construct, NC-vector, or miR-145-5p mimic and RAB18 construct. (**p < .01, *p < .05). miR-145-5p indicates microRNA-145-5p; HCC, hepatocellular carcinoma; NC-vector, negative control vector.

Authors’ note
Written informed consent was obtained from all individual participants included in the study. Baoying Wang and Wenjing Dong contributed equally to this work.

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