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MiR-876-5p acts as an inhibitor in hepatocellular carcinoma progression by targeting DNMT3A

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the biggest challenges that human beings faced with in 21st century. Previous researches have revealed that miRNAs can serve as regulators in various cancers. MiR-876-5p, a member of miRNA family, has been studied in lung cancer for its anti-oncogenic function. However, the exact function of it is not reported in HCC. Our study aims to find out the effects of miR-876-5p expression on HCC progression. Two HCC cells were chosen to do functional assays after miR-876-5p expression was detected in cell lines by qRT-PCR. HepG2 cell was transfected with miR-876-5p mimics, whereas LM3 cell was transfected with miR-876-5p inhibitors. Next, cell activities of these two indicated cells were analyzed by means of MTT assay, colony forming assay, transwell migration assay and western blot analysis. Consequently, we found that miR-876-5p could inhibit both cell proliferation and metastasis. Moreover, we found out a target gene (DNMT3A) of miR-876-5p by performing bioinformatics analysis, dual luciferase reporter assay and biotin-avidin pull-down assay. Finally, rescue assays were carried out in HepG2 cells. We found that DNMT3A could reverse miR-876-5p mimics-induced inhibition. Therefore, we concluded that miR-876-5p suppressed hepatocellular carcinoma progression by targeting DNMT3A.

1. Introduction

As one of the commonest primary liver cancer, hepatocellular carcinoma (HCC) has become the second major cause of cancer-related deaths all over the world [1,2]. Although researchers are unceasingly making progress in developing targeted therapeutics and immunotherapy strategies [3,4], sorafenib remains the principal one FDA-approved molecular inhibitor for advanced HCC. Hence, it is extremely urgent to decipher the novel potential biomarkers in carcinogenesis of HCC. Similarly, findings of novel biomarkers enable researchers to develop novel targeted therapeutics [5]. This study is dedicated to find out novel molecular targets for HCC.

As a sub classification of non-coding RNA, microRNAs (miRNAs) are composed of 14–24 nucleotides. It is reported that miRNAs can modulate gene expression at the post-transcriptional level. Moreover, miRNAs are known for their special biological functions [6]. For instance, miRNAs can act as regulators during tumorigenesis. miRNAs can be divided into two types (tumor facilitator and tumor suppressor) due to the different functions [7]. Over the past few years, more and more novel miRNAs have been discovered in progression and development of human cancers. MiRNAs have been proved to affect cell proliferation, apoptosis and migration of malignant tumors [8–10]. In these processes, many miRNAs undergo expression changes, thus modulating their downstream target genes to affect tumor phenotypes [11–13]. In recent years, miR-876-5p has been reported in human lung cancer for its anti-oncogenic functions [14]. However, the underlying molecular mechanism of miR-876-5p which affects hepatocellular carcinoma progression is almost unknown. Therefore, it is necessary to explore the expression pattern and biological role of miR-876-5p in HCC.

As an oncomRNA, DNMT3A has been verified to be a downstream target gene of some miRNAs in human cancers [15–17]. In this study, we tried to find the interaction between miR-876-5p and DNMT3A. We demonstrated the interactive pattern of them through performing bioinformatics analysis, luciferase reporter assay and pull-down assay. The effects of miR-876-5p-DNMT3A axis in HCC progression was certified by performing rescue assays. In conclusion, we proved that miR-876-5p is a tumor inhibitor in HCC progression via targeting DNMT3A.

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2. Materials and methods

2.1. Clinical samples

Firstly, we acquired human 62 pairs of HCC tissues and adjacent normal tissues from patients diagnosed with HCC in Fourth Department of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University. All patients participated in this study had signed the ethics consents before all experiments were conducted. In addition, this study had acquired the approval of the ethic committee of Eastern Hepatobiliary Surgery Hospital, Second Military Medical University.

2.2. Cell culture

The American Type Culture Collection (ATCC, Manassas, Virginia) is the institute from which we purchased human HCC cell lines (HepG2, Huh-7, SMMC7721, Hep3B, LM3, MHHC97H) and the normal liver epithelial cell (L-02). All cells were cultivated in Dulbecco modified Eagle medium (DMED; Thermo Fisher Scientific, Waltham, Massachusetts) by an addition of a mixture [(10% FBS (HyClone, Logan, Utah), penicillin (100 U/mL), and streptomycin (100 mg/mL)]. Cells were preserved in a moist air of 37 ˚C and 5% CO_2.

2.3. Cell transfection

To overexpress and knock down miR-876-5p, cells were separately transfected with miR-876-5p mimic and miR-876-5p inhibitor. Relatively, NC for miR-876-5p mimic and NC for miR-876-5p inhibitor were taken as the negative controls. All the above plasmids were synthesized and purified by Molbase Co Ltd, Shanghai, China. Additionally, we purchased the plasmids (pcDNA-KCNQ1OT1 and sh-KCNQ1OT1) from GenePharma (Shanghai, China) for KCNQ1OT1 overexpression and knockdown. Based on the instructions, Lipofectamine2000 (Invitrogen) was used to finish all transfections.

2.4. RNA extraction and qRT-PCR

TRIZOL (Invitrogen, Carlsbad, California) was used to extract total messenger RNAs (mRNAs) from HCC cells. PrimeScript RT reagent kit (TaKaRa, Dalian, China) was utilized for reverse transcription of complementary DNA synthesis. Next, to accomplish quantitative real-time polymerase chain reaction (qRT-PCR), miSYBRGreenPCR kit (TransGen Biotech, China) was applied. In this experiment, to normalize the expression of miR-876-5p, U6 snRNA was taken as an endogenous control.

Similarly, to identify the normalized expression of DNMT3A, beta-actin was regarded as the internal reference. The 2^{-DDCt} method was used to quantify the relative expression of miR-876-5p and DNMT3A.

2.5. Cell viability assay

After HCC cells were subjected to necessary transfection, they were cultivated at 37 ˚C and 5% CO_2 for 24 h. 20 mL of MTT were then put into each well, and cultured for another 4 h. Next, the cell supernatants were removed and thrown away. At that time, 150 mL of DMSO was poured into to each well. To dissolve the crystals, the plates were shaken for a quarter of an hour. Finally, the absorbance of each samples was determined at 570 nm on an ELISA microplate reader.

2.6. Colony formation assay

To further detect cell proliferation, cells (8×10^3) were suspended in 1.5 mL DMEM in which 10% fetal calf serum (FBS) had been added. Cells were incubated in a moist air containing 5% CO_2 at 37 ˚C. The incubation continued for 12 days until colonies could be seen visibly. At last, the colonies were fixed with ethanol and stained with crystal violet. We calculated the number of colonies under a Nikon Eclipse TS100 microscope (Nikon, Shinagawa, Japan).

2.7. Cell migration assay

Transwell chamber (8 um pore size, Corning) was utilized to measure the migrating ability of HCC cells. Briefly, the transfected cells (1×10^5 cells/well) were placed into the upper chamber. At the same time, the lower chamber was added into a kind of media which containing 10% FBS. After incubation for two days, we wiped off cells remaining in upper membrane thus to enable the migrating cells were fixed in methanol. Next, 0.1% crystal violet was used to stain cells so that we could calculate them under a microscope.

2.8. Dual luciferase reporter assay

The HepG2 and LM3 cells were co-transfected with wild-type or mutant 3¢-UTR sequence of DNMT3A and NC mimic or miR-876-5p mimic by Lipofectamine 2000 (Invitrogen). The luciferase activity was measured by performing Luc-PairmiR Luciferase Assay (GeneCopoeia, Rockville, Maryland).

2.9. Pull-down assay

At first, miR-876-5p, miR-876-5p-Mut, and miR-876-5p-NC were separately biotinylated to be bio-miR-876-5p-wt, bio-miR-876-5p-mut, and bio-NC by utilizing GenePharma Company (Shanghai, China). Next, they were transfected into HCC cells. 48 h later, cells were collected and lysed. They were then incubated with Dynabeads M-280 Streptavidin (Invitrogen, CA) for 10 min and washed with buffer. The bound RNAs were quantified and analyzed by qRT-PCR.

2.10. Western blot analysis

In order to extract total RNA, cells were lysed in an ice-cold RIPA buffer (Solaibo, China) at 48 h after transfection. BCA Protein Assay kit (Vigorous Bio-technology Beijing Co Ltd, Beijing, China) was then utilized for detection of total protein concentration. Next, proteins were transferred to PVDF membranes (Bio-Rad, Hercules, California) after they were isolated by SDS-PAGE. Proteins were blocked with 5% nonfat milk for one hour at normal temperature and incubated all night with primary antibodies (DNMT2A, E-cadherin, ZO-1, N-cadherin, Vimentin). They were diluted at a ratio of 1:1000. All these antibodies were purchased from Sigma-Aldrich Co Ltd (St. Louis, Missouri). Next, incubation with secondary antibodies [anti-IgG, (1:2000 dilution); Zhongshan Biology Company, Beijing] was performed. Finally, the ImageJ software (National Institutes of Health, Bethesda, Maryland) was used to quantify the protein bands.

2.11. Statistical analysis

Statistical analyses were made by using SPSS Statistics 18.0 (IBM, Armonk, NY, USA). Data are displayed as mean ± SD. Two group comparison and multiple group comparison were calculated with Student's *t*-test and one-way ANOVA. Kaplan-Meier method helped us to plot the overall survival curves. Cox proportional hazards regression model was generated to identify elements closely correlated with overall survival of HCC patients. The correlation between miR-876-5p and DNMT3A was analyzed by Spearman correlation test. P-value < 0.05 indicated differences have statistically significance.

3. Results

3.1. MiR-876-5p down-expression is a prognostic factor in HCC

*In order to investigate the exact role of miR-876-5p in HCC, we