Role of miR-193a-5p in the proliferation and apoptosis of hepatocellular carcinoma

J.-T. WANG¹, Z.-H. WANG²

¹Department of Hepatobiliary Surgery, Yantai Yantaishan Hospital, Yantai, China
²Department of Gastroenterology, Yantai Yuhuangding Hospital, Yantai, China

Abstract. – OBJECTIVE: This study aims at exploring the regulatory effects of miR-193a-5p on hepatocellular carcinoma (HCC). It might provide new insight into the improvement of clinical treatment of HCC.

PATIENTS AND METHODS: A total of 50 HCC patients who did not receive any tumor treatments were recruited, and 50 paired tumor tissues and adjacent non-tumor tissues were obtained. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to validate the expression and significance of miR-193a-5p in HCC tumor tissues, adjacent non-tumor tissues and cell lines. Binding-site of the target gene of miR-193a-5p was predicted by bioinformatics and further verified by Dual-Luciferase reporter gene assay and Western blotting (WB) assay. To investigate the potential role of miR-193a-5p in HCC development, cell counting kit-8 (CCK-8) assay was performed to study the proliferation and viability capacities. Flow cytometric analyses were adopted to test the cell cycle distribution and quantify the apoptotic cell proportion.

RESULTS: MiR-193a-5p expression was specifically up-regulated in HCC tissues and cell lines compared with paired adjacent non-tumor tissues and normal liver cell lines (HL-7702) respectively. BMF was considered as a downstream gene of miR-193a-5p, which was further proofed in Dual-Luciferase reporter gene assay and Western blot assays. In vitro experiments showed that miR-193a-5p overexpression could accelerate the proliferation, facilitate the G1/S transition and suppress the apoptosis of HCC cells. However, BMF overexpression could reverse the effects of miR-193a-5p on the cellular functions of HCC cells.

CONCLUSIONS: This finding suggested that miR-193a-5p is strongly up-regulated in HCC. MiR-193a-5p promoted the abnormal proliferation of HCC cells and limited their apoptosis by targeting the downstream gene BMF. Thus, the miR-193a-5p/BMF axis might be a novel regulatory pathway of apoptosis which could be potential therapeutic sites in HCC treatment.

Key Words: Hepatocellular carcinoma (HCC), MiR-193a-5p, Bcl-2 modifying factor (BMF), Proliferation, Apoptosis.

Introduction

Globally, the incidence and mortality rate of hepatocellular carcinoma (HCC), as one of the most common malignant tumors¹, rank 5th and 3rd in malignant tumors, respectively². According to statistics, there are about 740,000 new cases and 690,000 deaths of HCC throughout the world every year³. In China, the mortality rate of HCC ranks 2nd, and approximately 110,000 patients die of HCC every year⁴. HCC is characterized by hidden onset, difficulty in early diagnosis and poor prognosis, which has seriously threatened the people’s health. About 80% of HCC patients are already in an advanced stage at the time of diagnosis, and the 5-year survival rate for them is about 5%⁵. HCC is easily prone to metastasis and recurrence, which also seriously affects the therapeutic effects on HCC. The molecular mechanism in the occurrence, development, metastasis, and recurrence of HCC are complex and have not been fully clarified. Therefore, it is of great significance to study the molecular mechanism of HCC to improve the prevention, diagnosis, treatment and prognosis of HCC.

With the research progress in recent years in the medical field, especially the oncology field, several new treatment methods have emerged, some of which had been applied in the clinic, achieving satisfactory effects in tumor treatment. In these new treatment means, micro ribonucleic acid (miRNA) has been well concerned. MiRNA is a kind of small non-coding RNA with 19-25 nucleotides in length, which can block mRNA translation and/or negatively regulate its stability⁶. At the same time, there have been more than 500 different miRNAs
confirmed in human cells. Evidence has proved that the regulation of the miRNA level is extremely important for the normal growth and differentiation of a variety of cells and tissues.

MiR-193a-5p is a member of the miR-193 family, which is located on human chromosome 17q11.2. As a member of the miRNA family, miR-193a-5p can down-regulate the expression of the target gene through binding to the 3'-untranslated region (UTR) of the target gene mRNA. Reports showed that miR-193a-5p dysregulation would increase the malignant degree of tumor cells and enhance the proliferative, migratory and invasive capacities of tumor cells. However, whether miR-193a-5p is involved in the pathological progression of HCC remains unclear yet.

Patients and Methods

Tissue Samples and Cell Lines

50 paired tumor tissues and adjacent non-tumor tissues were harvested from 50 HCC patients undergoing surgical treatment in our hospital from July 2014 to September 2017. The adjacent non-tumor tissues were resected more than 5 cm from the edge of the tumor. All tissues were quickly frozen in liquid nitrogen for the following experiments. Enrolled patients did not receive preoperative radiotherapy or chemotherapy, and were pathologically diagnosed as HCC with negative paraneoplastic histopathology. After all, the Declaration of Helsinki should be mentioned and respected. This study was approved by the Ethics Committee of Yantai Yantaishan Hospital. Signed written informed consents were obtained from all participants before the study.

Human hepatocellular carcinoma cell lines (HepG2) and normal liver cell lines (HL-7702) were provided by Nanjing Kaiji Biotechnology Development Co., Ltd. (Nanjing, China). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and placed in a 37°C, 5% CO2 incubator. The fresh medium was replaced according to the cell growth density. Cells in logarithmic growth phase were taken for the experiment.

Target Gene Prediction

The target gene of miR-193a-5p was predicted using bioinformatics prediction software Targetscan and miRanda. The appropriate target genes were selected combined with differentially-expressed proteins.

Dual-Luciferase Reporter Gene Assay

HepG2 cells were inoculated into a 24-well plate. Transient transfection was conducted according to instructions until 60% of cell confluence. The miR-193a-5p mimic/NC and pmirGLO-BMF plasmid (wt)/(mut) were co-transfected in HCC cells, followed by culture for another 36 h. Luciferase activity was analyzed in accordance with instructions of the Dual-Luciferase activity assay kit using the multi-functional microplate reader.

Transfection

HepG2 cells were inoculated into a 6-well plate and cultured until the cell density was 50-70%. 10 μL of miRNA-NC was added into each well in NC group for transfection. 10 μL of miRNA-mimic was added into each well in mimic group. 10 μL of miRNA-mimic and siRNA BMF were transfected in cells of mimic + siRNA BMF group.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

The total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and miRNA was extracted from liver tissues using the special extraction kit, followed by reverse transcription. After complementary Deoxyribose Nucleic Acid (cDNA) obtained was diluted in an appropriate ratio, the qRT-PCR system was prepared according to instructions, with U6 used as an internal reference. Amplification was performed in the Bio-Rad IQ5 PCR instrument. Results in PCR were calculated using the 2-ΔΔCt method. Primer sequences used in this study were as follows: microRNA-193a-5p, F: 5'-TATATGGGTCTTTGC-GGCG-3', R: 5'-GTGCAGGGTCCGAGGT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTA-AAAT-3', R: 5'-CGCTTCAGAATTTGCGTGT-CAT-3'.

Western Blot Analysis

The RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein from cells 72 h after transfection. The BCA (bicinchoninic acid, Pierce, Rockford, IL, USA) method was performed to quantitate the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were
incubated with primary antibody BMF rabbit anti-human monoclonal antibody, 1:1000, (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody (HRP-labeled goat anti-rabbit antibody, 1:2000) after rinsing with TBS-T (Tris-buffered saline with Tween 20). Chemiluminescence was used to expose the protein bands on the membrane.

Cell Proliferation Detection
The growth curve was determined via cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). In brief, HepG2 cells were incubated into a 96-well plate (1 \times 10^3/100 μL/well), transfected with corresponding miRNAs after 24 h, and cultured for 24, 48 and 72 h, respectively. Cells were collected for detecting the absorbance at the wavelength of 450 nm according to the instructions of the CCK-8 kit, and the growth curve was plotted.

Cell Cycle Detection
After treatment for 48 h, cells were collected and their distribution was detected via flow cytometry in accordance with the instructions of the cell cycle detection kit, followed by result analysis.

Detection of Apoptosis via Flow Cytometry
The apoptosis of HepG2 cells in different groups was detected via flow cytometry. Cells were washed with phosphate-buffered saline (PBS), digested and prepared into cell suspension (1 \times 10^6/mL). After centrifugation, the supernatant was discarded and washed, suspended using 100 μL of binding buffer and mixed evenly with Annexin V-fluorescein isothiocyanate (FITC), followed by detection using flow cytometry within 1 h.

Statistical Analysis
All experimental data were presented as mean ± standard deviation (x ± s). The t-test and one-way analysis of variance were performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). The comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). p<0.05 was suggested as statistically significant. All experiments were repeated 3 times.

Results
Abnormal Expression of miR-193a-5p and BMF in HCC Tissues and Cells
We used qRT-PCR assay to determine the relative expression levels of miR-193a-5p in 50 pairs of HCC tissues and adjacent non-tumor tissues. The results showed that the expression level of miR-193a-5p in HCC tissues was significantly higher than that in adjacent non-tumor tissues (Figure 1A). Subsequently, qRT-PCR was performed on the level of miR-193a-5p in HCC cell lines (HepG2) and normal liver cell lines (HL-7702) (Figure 1B). Similarly, the results in-
dicated that the expression level of miR-193a-5p in HepG2 cells was significantly lower than that of HL-7702 cells.

Detection of MiR-193a-5p Mimics Transfection Efficiency

The transfection efficiency was detected by the qRT-PCR assay. As shown in Figure 2, the expression of miR-193a-5p in HepG2 cells was up-regulated after transfection with miR-193a-5p mimics. This result confirmed that the transfection of miR-193a-5p mimics could significantly increase the expression level of miR-193a-5p.

Target Gene Prediction Results

BMF was the target gene of miR-193a-5p, and the complementary base sequence of miR-193a-5p and BMF mRNA 3'-UTR predicted by the software was displayed in the underline (Figure 3A).

Dual-Luciferase Reporter Gene Assay Results

As shown in Figure 3A, the luciferase activity declined significantly when co-transfected with miR-193a-5p mimics and pmirGLO-BMF 3’-UTR (wt) plasmid compared with NC. After point mutation in base binding site predicted by the software, there was no significant change in Luciferase activity when transfected with pmirGLO-BMF 3’-UTR (mut) plasmid. It is indicated that miR-193a-5p could bind to the 3’-UTR of BMF, and BMF is a potential target gene for miR-193a-5p.

Figure 2. Transfection efficiency detection by qRT-PCR (**p < 0.01).

Figure 3. BMF was a direct and functional target of miR-193a-5p. A, Diagram of putative miR-193a-5p binding sites of BMF and relative activities of Luciferase reporters (**p < 0.01). B, MiR-193a-5p decreases the expression level of BMF detected by Western blot experiment (***p < 0.001 vs. NC group; **p < 0.01 vs. mimics group).
**MiR-193a-5p Decreased the Expression Level of BMF**

In Western blot assays, we also found that BMF expression was significantly lower in HepG2 cells transfected with miR-193a-5p mimics. These data further illustrated the regulatory effects of miR-193a-5p on BMF expression (Figure 3B).

**MiR-193a-5p Accelerated the Cell Proliferation**

Results of CCK-8 assay revealed that the absorbance of HepG2 cells escalated and the proliferation speed increased after transfection with miR-193a-5p mimics. After BMF overexpression in cells, the absorbance of tumor cells decreased and the proliferation speed was reduced significantly (Figure 4A). Further, it was found via flow cytometry that after transfection with miR-193a-5p mimics in HepG2 cells, the proportion of cells in G0/G1 phase significantly decreased, while that in S phase increased. However, after BMF overexpression in HepG2 cells, miR-193a-5p-induced G1/S transition could be alleviated significantly, manifesting as the decreased number of cells in S phase and the inhibited conversion from G0/G1 phase to S phase (Figure 4B).

**MiR-193a-5p Inhibited the Apoptosis of HepG2 Cells**

Flow cytometry analysis showed that in the miR-193a-5p mimics group, the apoptosis rate of HepG2 cells was significantly lower than that of the control group, as shown in Figure 5. However, after co-transfection of miR-193a-5p mimics and siRNA BMF, the apoptosis rate of HepG2 cells significantly increased compared with cells transfected with miR-193a-5p mimics only. Thus, miR-193a-5p was found to regulate apoptosis in HepG2 cells by targeting BMF.

**Figure 4.** MiR-193a-5p accelerated the cell proliferation and facilitated the G1/S transition of HCC cells. **A,** Cell proliferation was detected by CCK-8 assay (**p < 0.001**). **B,** The cell cycle phases of HCC cells were analyzed using flow cytometry (**p < 0.01 vs. NC group; *p < 0.05, **p < 0.01 vs. mimics group).**

**Figure 5.** MiR-193a-5p/BMF axis suppressed the apoptosis of HCC cells. Apoptosis level of HepG2 cells was tested by flow cytometry. All data were presented as means ± standard deviations (*p < 0.05 vs. NC group; #p < 0.05 vs. mimics group).
Discussion

Expression dysregulation of miRNA is a common characteristic in both solid and hematological malignant tumors, and there is strong evidence proving that miRNA plays a role as an oncogene or tumor-suppressor gene. MiRNA has a small molecular weight and a small proportion in the genome, but they exert a potent regulatory effect in cells. Currently, studies mainly focus on the interaction between miRNA and mRNA in the 3'-UTR in target gene mRNA. Each miRNA contains more than 20 target regions of the target gene. Even though the proportion of miRNA transcriptional gene in the genome is as small as 1-5% in all transcripts, up to 30% of its functional genes can be affected. According to different functions of the target genes, miRNA can be involved in regulating the cell cycle, differentiation, apoptosis and other functions. Increasing studies have found that miRNA, in addition to regulating physiological functions, plays a crucial role in tumor development. MiRNA plays a role as an oncogene or tumor-suppressor gene in tumors, so the function of miRNA has become a research hotspot recently.

The discovery of miRNA broadens our knowledge of the complex regulation of gene expression. Dysregulation of miRNA leads to abnormal regulation of target gene, further affecting various diseases. In liver diseases, miRNA is closely correlated with liver fibrosis, inflammation and liver tumor. It is of great significance to understand the role of miRNA and target genes in these liver diseases, especially HCC.

BMF, a member of the Bcl-2 family, is one of the most common pro-apoptotic proteins in the tumor. BMF is located on chromosome 15q14 in the human body and widely expressed in a variety of tissues. The pro-apoptotic effect of BMF has been confirmed in various cells. BMF is bound to the dynein complex in normal cells and maintains a resting state. After dissociation from the dynein complex, BMF is involved in mitochondrial apoptosis pathway. The abnormal expression of BMF would block the apoptosis pathway and increase the cell proliferative capacity. Currently, some studies have demonstrated that BMF plays an important role in the malignant progression of tumor cells. Guo et al found that BMF, which could recognize STARD13 3'-UTR, promotes apoptosis in breast cancer. Besides, as a functional target, Xia et al found that miR-125b promotes human glioma cell proliferation and inhibits ATRA-induced cell apoptosis by targeting BMF.

According to previous studies, miR-193a-5p was abnormally expressed in different kinds of tumors. In this study, the expression level of miR-193a-5p in HCC tissues and adjacent non-tumor tissues of 50 HCC patients was detected. It was found for the first time that miR-193a-5p is significantly up-regulated in HCC, indicating that miR-193a-5p is possibly involved in the occurrence and development of HCC. According to the prediction using bioinformatics software, BMF was the target gene of miR-193a-5p. Moreover, Dual-Luciferase reporter gene assay proved that miR-193a-5p could act on BMF 3'-UTR directly without influence on the BMF 3'-UTR mutant. It was further verified via Western blotting in HCC cell lines (HepG2) that transfection of miR-193a-5p mimics could reduce the protein expression of BMF in HCC cells, proving that BMF was a direct target gene of miR-193a-5p. In addition, miR-193a-5p up-regulation could accelerate the proliferation, facilitate the G1/S transition and suppress apoptosis of HCC cells. The above cell functions were effectively reversed after overexpression of BMF in cells, indicating that miR-193a-5p up-regulation in HCC might promote abnormal proliferation, cell cycle and anti-apoptotic changes through regulating BMF.

Conclusions

Nowadays, the functions of miRNAs, as well as their target genes, have great potential to serve as new targets and directions for treatment of HCC. We provide a new clue for clarifying the pathogenesis of HCC, indicating miR-193a-5p/BMF might be a new therapeutic target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

Role of miR-193a-5p in the proliferation and apoptosis of hepatocellular carcinoma


