MiR-5692a promotes proliferation and inhibits apoptosis by targeting HOXD8 in hepatocellular carcinoma

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Summary

Purpose: Hepatocellular carcinoma (HCC) is a member of the most frequent malignancies in the world and the poor prognosis of HCC is mainly due to lack of early detection and treatment. The purpose of this study was to investigate the role of microRNA (miR)-5692a in the progression of HCC and its underlying mechanism.

Methods: The relative expression of miR-5692a in HCC tissues and cell lines was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) assay. Cell counting kit-8 assay and colony formation assay were used to determine cell proliferation. Flow cytometric analysis was carried out to determine cell cycle distribution and apoptotic cells. Bioinformatics analysis and dual luciferase reporter assay were employed to predict and verify the potential targets of miR-5692a. Protein expression level of HOXD8 was assessed by western blotting normalized by GAPDH in transfected cells.

Results: The relative expression level of miR-5692a was increased in both HCC tissues and cell lines. According to CCK8 assay and colony formation assay, miR-5692a was considered to promote proliferation in HCC. The consequence of flow cytometric analysis showed that overexpressed miR-5692a accelerated cell cycle and inhibited cell apoptosis. We verified that HOXD8 was a target of miR-5692a via online prediction database and dual luciferase reporter assay. The rescue assay we carried out subsequently validated that miR-5692a functioned as an oncogene by regulating HOXD8 in HCC.

Conclusions: This study revealed that miR-5692a had an oncogenic role in HCC by targeting HOXD8 which might bring a novel insight into new therapeutic targets and biomarkers in HCC.

Key words: apoptosis, HOXD8, MiR-5692a, proliferation

Introduction

Hepatocellular carcinoma (HCC) is the prevailing histological type in liver cancer, and a frequent malignant disease in the world [1]. According to the cancer statistics, death rates of 4 major cancers (lung, breast, prostate, and colorectum) show a significant declining trend. By contrast, death rates of liver cancer rose from 2010 to 2014 by nearly 5% per year [1]. As a recent study reported, only about 10-30% of patients have the opportunity for liver resection or liver transplantation [2]. The poor prognosis of HCC is mainly due to lack of early detection and efficient therapy.

MicroRNAs (miRs) are small, endogenous, noncoding RNAs, which are proved to have essential effect on upregulating and downregulating the target genes by directly binding to its 3’ untranslated regions (3’ UTRs) [3]. Mounting evidence showed that miRs function as oncogenes or tumor suppressors in various cancers [4-6]. Numerous studies have shown that miRs take part in the cell progression...
including differentiation, proliferation, apoptosis and cell cycle in cancer development [7-9]. However, miR-5692a has rarely been researched. Hence, the study on miR-5692a may provide a novel insight into therapy and biomarker in HCC.

To the best of our knowledge, miRs occupy an essential position in the progression of malignancies by regulating the downstream target genes. Thus, we carried out bioinformatics analysis to figure out the underlying targets of miR-5692a. From the results of bioinformatics analysis, HOXD8 (homeobox D8) emerged with high context++ scores. HOXD8 is a member of HOX genes, which are deregulated in various cancers, including colorectal carcinoma, HCC, and lung cancer [10-12]. As reported, HOXD8 is significantly correlated with proliferation, cell apoptosis and cell cycle.

In this study, we showed that miR-5692a was upregulated in both HCC tissues and cell lines. By bioinformatics analysis and dual luciferase reporter assay, we confirmed that HOXD8 was a downstream target gene of miR-5692a. Through this research, we validated that miR-5692a acted as an oncogene in the development of HCC by regulating HOXD8 expression.

Methods

This research was approved by the ethics committee of Yantai Yuhuangding Hospital. All participants provided written informed consents before the study entry.

Tissue specimens

All the tissue specimens in this study were obtained from patients in Yantai Yuhuangding Hospital during 2016-2017. Sixty paired specimens paired of HCC and para-tumor samples were examined for the relative expression level of miR-5692a by quantitative real-time polymerase chain reaction (qRT-PCR). HCC specimens and para-tumor tissues were subsequently deposited in liquid nitrogen before used.

Cell culture

The HCC cell lines Huh-7, Hep3B, SMMC-7721, MHCC-97H, HepG2 and the normal hepatocyte LO2 were purchased from the Institute of Biochemistry and Cell Biology (Beijing, China). These cells were cultured in Roswell Park Memorial Institute 1640 (RPIM1640, HyClone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100U/mL penicillin and 100ug/mL streptomycin (HyClone, South Logan, UT, USA) at 37°C with 5% CO2.

Cell transfection

For the overexpression of miR-5692a, oligonucleotides obtained from GenePharma (Shanghai, China) including negative control (NC), miR-5692a mimics (mimics), inhibitor negative control (inhibitor NC) and miR-5692a inhibitor (inhibitor) were employed in this study. The transfection With negative control (NC), miR-5692a mimics (Mimics), negative control inhibitor (inhibitor NC) and mir-5692a inhibitor (inhibitor) of designated cell lines was performed via Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The siRNA transfection reagent (Invitrogen, Carlsbad, CA, USA) was used following the manufacturer’s instructions. The efficiency of cell transfection was determined by qRT-PCR.

RNA extraction and qRT-PCR

All cell lines and specimens were prepared to extract total RNA by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) on the basis of the manufacturer’s protocol. The Reverse Transcription Kit (TaKaRa, Dalian, China) was brought in to synthesize cDNAs. The relative expression level of miR-5692a and HOXD8 was then determined by qRT-PCR and GAPDH was used to normalizing the data. qRT-PCR was used in the ABI 7500 system (Applied Biosystems, Foster City, CA, USA).

 Colony formation

1.5×10^4 cells were planted into cell culture plates (60mm) and cultured for 2 weeks. Cells were then washed twice by phosphate buffer saline (PBS) (Beyotime, Shanghai, China) and fixed in ice cold 70% methanol for 15 min and Crystal Violet Staining Solution (Beyotime, Shanghai, China) was used to stain the cell colonies. All the colonies were subsequently photographed.

Cell-counting kit-8 assay

Furthermore, cell counting kit-8 (CCK-8) assay was performed to elucidate the influence of miR-5692a on proliferation. Transfected cells were planted into 96-well plates (7×10^3/well) and then CCK-8 solution (Beyotime, Shanghai, China) (10µL/well) was used to stain cells for 2 hrs at 37°C. The optical density (OD) value (450nm) was evaluated with spectrophotometer (Thermo Scientific, Rockford, IL, USA).

Flow cytometric analysis

For studying apoptosis, the Annexin V-FITC/prodium iodide (PI) Apoptosis Detection Kit (Vazyme, Nanjing, China) was used following the manufacturer’s protocol. To monitor the cell cycle, transfected cells were immersed in cold 70% ethanol overnight before stained with PI (Vazyme, Nanjing, China). The flow cytometric analysis took place at BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ,USA) flow cytometry.

Bioinformatics analysis

To seek for the underlying target genes of miR-5692a, we used the 4 public available algorithms: RNA22, TargetScan, miRWalk and MiRanda. The results suggested that 3′-UTR of HOXD8 has potential binding sites with miR-5692a. Thus, we considered that HOXD8 may be served as a target of miR-5692a.

Luciferase report assay

Wild HOXD8 3′-UTR sequence or the mutant HOXD9 3′-UTR sequence was combined top GL3
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promoter vector (Genscript, Nanjing, China). Luciferase activity was determined in Victor 1420 Multilabel Counter (Wallac, Finland) by Luciferase Assay System (Promega, Hercules, CA, USA) following the manufacturer’s protocol.

Western blot

Total protein was isolated by RIPA buffer (Thermo, Waltham, MA, USA) and phenylmethanesulfonyl fluoride (PMSF). Protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Then, the membrane was immunostained at 4°C by rabbit anti-HOXD8 (1:1000, CST, Danvers, MA, USA) overnight. Rabbit anti-GAPDH (1:5000, CST, Danvers, MA, USA) was taken as a loading control. Protein relative expression level was evaluated by Image J software.

Statistics

All experiments in this study were performed in triplicate. All data recorded were presented as mean±standard deviation (SD). Pearson’s correlation test was performed for correlation analysis. Student’s unpaired t-test was used to compare the variables between two groups. In this study, p<0.05 signified statistical significance.

Results

MiR-5692a was upregulated in HCC

The relative expression level of miR-5692a in HCC tissues and cell lines was evaluated by qRT-PCR. miR-5692a was up-regulated in HCC tissues compared to para-tumor tissues (Figure 1A). Besides, in 5 HCC cell lines, the expression level of

Figure 1. The expression of miR-5692a showed in tissues and transfected cells. A: The expression of miR-5692a was detected in 60 paired HCC tumor and para-tumor tissues. B: qRT-PCR was used to verify the expression of miR-5692a in HCC cell lines. C: Expression of miR-5692a in the Huh-7 cell line after transfection with mimic. D: Expression of miR-5692a in the HepG2 cell line after transfection with inhibitor. **p<0.01, ***p<0.001, compared to control group. The data are expressed as mean ± SD.
miR-5692a was relatively higher when compared with normal hepatocyte LO2 (Figure 1B). Taken together, all the results suggested that miR-5692a was positively correlated with the progression of HCC.

Based on the results above, we constructed 4 oligonucleotides expressing NC, mimics, NC inhibitor, and miR-5692a inhibitor. To investigate the biological function of miR-5692a in HCC progression, Huh-7 was transfected with NC and mimics while HepG2 was transfected with NC inhibitor and miR-5692a inhibitor. QRT-PCR was then employed to test the expression of miR-5692a in the transfected cell lines. The results demonstrated that miR-5692a was upregulated after transfection with mimics in Huh-7 cell line (Figure 1C) and was downregulated when transfected with inhibitor in HepG2 cell line (Figure 1D).

**MiR-5692a promoted cell proliferation and inhibited apoptosis in HCC cell lines**

CCK-8 assay was used to evaluate cell proliferation post-transfection. As shown in Figure 2A, the OD value was higher in the mimics group which revealed higher proliferation in Huh-7 cell line.

**Figure 2.** MiR-5692a contributed to cell proliferation. A: CCK-8 was used to analyze the cell proliferation and higher OD value represented increased cell proliferation. B: Cell colonies were stained by crystal violet and the number of colonies was counted, and the results of cell colonies showed that upregulation of miR-5692a accelerated significantly the formation of colonies, while downregulated miR-5692a inhibited cell formation compared to the control group. The time for cell colony formation was 10 days. **p<0.01,***p<0.001, compared with control group. The data are expressed as mean ± SD.
while the proliferation was inhibited in the inhibitor group in HepG2 cell line. Besides, colony formation showed that upregulation of miR-5692a significantly accelerated the formation of colonies. On the contrary, downregulated miR-5692a inhibited cell formation compared to the control group (Figure 2B). These results confirmed that miR-5692a was closely correlated with cell proliferation.

For further validation, flow cytometry was performed to examine cell cycle and cell apoptosis. As shown in Figure 3A, the cell cycle was accelerated with upregulated miR-5692a expression, indicating active status on cell proliferation. On the other hand, the cell viability was impaired after inhibition of miR-5692a (Figure 3B). The above findings showed that miR-5692a was overexpressed in HCC cell lines and played an essential role in cell proliferation and apoptosis.

HOXD8 was a direct target of miR-5692a in HCC cell lines

RNA22, TargetScan, miRWalk and MiRanda were employed to predict the target gene of miR-5692a. HOXD8 was predicted as a potential tar-

![Figure 3. MiR-5692a contributed to cell proliferation and negatively regulated apoptosis. A: Cell cycle was detected by flow cytometry. B: The rate of living cells was examined by flow cytometry.*p<0.05, **p<0.01, ***p<0.001 compared with control group. The data are expressed as mean ± SD.](image-url)
Figure 4. HOXD8 was proved to be the target gene of miR-5692a. A: Luciferase reporter assay was constructed to prove that miR-5692a directly bound to the 3'-UTR region of HOXD8. The sequence of wild-type and mutant types in HOXD8 3'-UTR is shown. After co-transfection with wild-type or mutant 3'-UTR reporter plasmids, the relative HOXD8 luciferase activity was calculated and recorded in histogram. B: The expression of HOXD8 in HCC tumor tissues and para-tumor tissues. C: SPSS was used to analyze the correlation between the mRNA level of HOXD8 and the expression of miR-5692a (p<0.001). D: HOXD8 was chosen from several databases. qRT-PCR was used to examine the mRNA of HOXD8 in transfected cell lines. E: Western blot was used to test the protein level of HOXD8 in transfected cell lines. **p<0.01, ***p<0.001, compared with control group. The data are expressed as mean ± SD.
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To target miR-5692a and verify the prediction, we performed a 3’-UTR assay. The results showed that the luciferase activity level was significantly lower than the NC group after co-transfection with HOXD8 WT 3’-UTR. In contrast, there was no significant change in luciferase activity after the co-transfection of mutant 3’-UTR form (Figure 4A), where the results revealed that miR-5692a could directly bind to the 3’-UTR of HOXD8.

For further validation, we used qRT-PCR to quantify the expression of HOXD8 in HCC tissues and found that its expression was higher in HCC para-tumor tissues compared to tumor tissues (Figure 4B). What’s more, miR-5692a expression was negatively correlated with the HOXD8 level in HCC tissues, which is consistent with our hypothesis (Figure 4C). qRT-PCR was also used to verify the mRNA expression of HOXD8, as shown in Figure 5.

**Figure 5.** MiR-5692a regulates cell proliferation and viability by directly targeting HOXD8. A: qRT-PCR was used to examine the mRNA of HOXD8 in cells transfected with siRNAs. B: qRT-PCR was used to examine the mRNA of HOXD8 in inhibitor-transfected cells transfected with si-HOXD8 NC and si-HOXD8. C: CCK-8 was used to analyze cell proliferation and higher OD value showed higher cell proliferation. D: Flow cytometry was used to examine cell cycle. E: Flow cytometry was used to detect the apoptotic rates. *p<0.05, **p<0.01, ***p<0.001, compared to control group. The data are expressed as mean ± SD.
Role of MiR-5692a in HCC

MiR-5692a regulated cell proliferation and apoptosis by targeting HOXD8

To investigate whether miR-5692a regulated the cell biological functions by binding to HOXD8, we transfected HepG2 cells with si-HOXD8 NC and siRNAs. The results of qRT-PCR demonstrated that the relative expression level of HOXD8 was downregulated in three siRNA groups (Figure 5A). Then, we transfected miR-5692a inhibitor-transfected cells with si-HOXD8 NC or si-HOXD8. qRT-PCR was used to evaluate the relative expression of HOXD8 and the results showed the HOXD8 was lower-expressed in the si-HOXD8 group than in the control group (Figure 5B). Besides, we repeated CCK-8 assay and flow cytometric analysis. Compared with the control group, higher OD value was detected in CCK-8 assay in inhibitor-si-HOXD8 group. Meanwhile, the effects of cell cycle arrest and apoptosis promotion in the inhibitor group were abolished after co-transfection with si-HOXD8 (Figure 5C, 5D, 5E). Taken together, these findings revealed that miR-5692a downregulated HOXD8 expression level in the HCC cells and this contributed to the promotion of cell proliferation and inhibition of apoptosis in HCC.

Discussion

HCC is known as one of the most frequent malignant tumors worldwide and more than a half of the HCC new cases occur in China [13]. Numerous investigations about the etiology of this disease have revealed several factors, such as radiation, chemical exposure and hepatitis [14]. In addition to the diversity of the causes of HCC, death rate of HCC still shows an accelerating trend [1]. Though reasons are complex, the lack of early diagnosis and efficient treatment is the heart of the problem. Therefore, novel biomarkers and therapy strategies of HCC are needed to be clarified.

In recent years, miRNAs are studied by numerous researchers. Abundant data from various experiments aiming at miRNAs revealed that they play a vital role in the progression of malignancy. miR-661 was reported to promote cell invasion and metastasis in non-small lung cancer by regulating RB1 [15]. In another study, miR-139-5p was proved to inhibit aerobic glycolysis, cell proliferation, migration and invasion in hepatocellular carcinoma by interacting with ETS1 [16]. MiR-543 was considered to promote proliferation and epithelial-mesenchymal transition by regulating RKIP in prostate cancer [17]. As the searches move along, more and more functions of miRNAs in cancer development are reported, such as differentation, glycolysis, proliferation, invasion, and cell apoptosis, cell cycle, and cell senescence [18-22]. Although a large number of miRNAs has been studied, miR-5692a is rarely been searched. Hence, we carried out this research on miR-5692a to figure out whether it affected HCC progression. Through qRT-PCR, we proved that the relative expression level of miR-5692a was relatively higher in HCC tissues when compared with para-tumor tissues. Consistently, the relative expression level of miR-5692a in HCC cell lines was relatively higher than in normal hepatocytes. In a series of physiological assays in vitro, miR-5692a promoted proliferation and inhibited cell apoptosis. Taken together, all the results suggested that miR-5692a functions as an oncogene in HCC.

MiRNAs function as oncogenes or tumor suppressors by regulating target genes through directly binding to their 3'-UTR region [3]. Since miR-5692a is poorly studied, this study brought new information via bioinformatics analysis. Through this analysis, we predicted the potential downstream targets of miR-5692a and considered HOXD8 as a target gene of miR-5692a. To validate our hypothesis, dual luciferase reporter assay was employed and the result suggested that HOXD8 was a direct target of miR-5692a. HOXD8 is a member of HOX gene family and has been proved to be deregulated in various kinds of cancers [11,23]. HOX8 is verified to be downstream target of several miRNAs and its dysregulation can abolish the effects of miRNAs in cancers [12,24]. Nevertheless, the correlation between miR-5692a and HOXD8 has not been studied yet. We verified that HOXD8 was a downstream target of miR-5692a in HCC cell lines by interacting with ETS1 [16]. MiR-543 was considered to promote proliferation and epithelial-mesenchymal transition by regulating RKIP in prostate cancer [17]. As the searches move along, more and more functions of miRNAs in cancer development are reported, such as differentation, glycolysis, proliferation, invasion, and cell apoptosis, cell cycle, and cell senescence [18-22]. Although a large number of miRNAs has been studied, miR-5692a is rarely been searched. Hence, we carried out this research on miR-5692a to figure out whether it affected HCC progression. Through qRT-PCR, we proved that the relative expression level of miR-5692a was relatively higher in HCC tissues when compared with para-tumor tissues. Consistently, the relative expression level of miR-5692a in HCC cell lines was relatively higher than in normal hepatocytes. In a series of physiological assays in vitro, miR-5692a promoted proliferation and inhibited cell apoptosis. Taken together, all the results suggested that miR-5692a functions as an oncogene in HCC.

In summary, our study confirmed that miR-5692a promotes cell proliferation and inhibits cell apoptosis in HCC. HOXD8 was proved to serve as
a downstream target of miR-5692a by both bioinformatics analysis and luciferase reporter assay. Through the results of the rescue assay used in this study, we believe that miR-5692a functions as an oncogene by regulating HOXD8 in HCC. Thus we consider that this study may bring a better understanding for miR-5692a and put forward a novel biomarker and therapeutic target in HCC.

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Conflict of interests

The authors declare no conflict of interests.

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