RESEARCH ARTICLE

miR-27a as an Oncogenic microRNA of Hepatitis B Virus-related Hepatocellular Carcinoma

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Abstract

  microRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through post-transcriptional interactions with mRNA. miRNAs have recently emerged as key regulators of various cancers. Although miR-27a has been implicated in several other cancers, its role in hepatitis B virus-related hepatocellular carcinoma (HCC) is unknown. In this study, we showed miR-27a to be frequently up-regulated in HCC tissues and HCC cell lines (HepG2 and Huh7). Overexpression of miR-27a enhanced cell proliferation, promoted migration and invasion, and activated cell cycling in HepG2 and Huh7 cells. In summary, our results suggest that up-regulation of miR-27a may play an oncogenic role in the development of HCC and might thus be a new therapeutic target in HCC patients.

Keywords: Hepatocellular carcinoma - microRNA - miR-27a - proliferation - migration and invasion

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and represents the third-leading cause of cancer-related death worldwide (El-Serag et al., 2008; Jemal et al., 2011). The vast majority of cases of HCC occur in the setting of liver cirrhosis caused by chronic infection with hepatitis B (HBV) and C (HCV) viruses and alcoholic liver disease (Tinkle et al., 2012). At present, the sensitivity and specificity of a-Fetoprotein (AFP) for diagnosis of HCC are not satisfying (Zinkin et al., 2008). Biomarkers for early detection and follow-up of HCC are absent, accounting for late diagnosis and subsequent poor prognosis. The mainstay of of treatment for HCC is surgical resection or liver transplantation. However, the recurrence rate of HCC after surgery is about 70%. Therefore, it is necessary to improve our understanding of the molecular pathogenesis of HCC and develop new target therapies and diagnostic biomarkers.

One of the most modern and progressive approaches for molecular characterization of various tumors today is based on micoRNA (miRNA) expression profiles. miRNAs, a group of small non-coding RNAs of about 22 nucleotides in length, is recently discovered and shown to regulate gene expression at the post-transcriptional level, by binding through partial sequence homology, to the 3'untranslated region (3'UTR) of mammalian target mRNAs and causing translational inhibition and/or mRNA degradation (Bartel, 2004). Increasing evidences show that miRNAs can act as oncogenes and tumor suppressors depending on tissue type and specific targets (Garzon et

al., 2009; Kasinski et al., 2011). Several studies have demonstrated miRNAs in human serum or plasma are very stable (Chen et al., 2008; Mitchell et al., 2008; McDonald et al., 2011). Specific circulating miRNAs were found to be potential diagnostic, prognostic, or metastatic markers with very high validity (sensitivity and specificity) (Huang et al., 2010; Brase et al., 2011; Zheng et al., 2011; Zhou et al., 2011; Liu et al., 2012; Schrauder et al., 2012). Recently, seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) in plasma were combined as a diagnostic panel, which was found to be a better diagnostic marker to distinguish HCC and healthy controls (Zhou et al., 2011). Low expression level of miR-27a in plasma was observed in patients with HCC compared with control groups of healthy, chronic hepatitis B, liver cirrhosis (Zhou et al., 2011). However, miR-27a has been found to be an oncogene, which is abnormally up-regulated in several types of cancers (Mertens-Talcott et al., 2007; Chintharlapalli et al., 2009; Guttilla et al., 2009; Liu et al., 2009). Thus, it remains to be determined whether miR-27a is altered in HCC and adjacent non-tumor tissues. Here, we report that miR-27a is significantly up-regulated in HCC tissue samples. In addition, we explored the biological significance of down-regulation and up-regulation of miR-27a using HCC cell lines in vitro.

Materials and Methods

Patient samples

  Twenty-four human HCC and adjacent non-tumor

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Table 1. Patient Characteristics

Factor	Values
Number of patients	24
Age	54.6±13.8
Sex	
male	19
female	5
Histological grade	
well-differentiated	9
moderately-differentiated	13
poorly-differentiated	2
TNM	
I	6
II	10
III	8

(liver) samples were collected at the time of surgical resection at the First Affiliated Hospital of Xingxiang Medical University between September 2010 and July 2012. Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. All patients have been infected by HBV. None of patients had received pre-operative chemotherapy, radiotherapy, transarterial chemoembolization or ablation. Both cancerous and non-cancerous specimens were histologically confirmed. The relevant characteristics of the studied subjects were shown in Table 1. This research was taken under consent of all patients for the use of their samples. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University. Written informed consent was obtained from all participants.

Cell lines

Human HCC cell lines HepG2 and Huh7 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin. All cell lines were incubated at 37°C in 5% CO_2.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen Corporation, California, USA) according to the manufacturer's protocol. To quantify mature miR-27a expression, the isolated total RNA was polyadenylated and reverse transcribed for use in a two-step quantitative RT-PCR. The complementary DNA from the miRNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) and an reverse transcript primer from RiboBio (Guangzhou, China). Real-time PCR analyses were performed with Real MasterMix (SYBR Green, Invitrogen) using synthesized primers that were purchased from RiboBio. U6 small nuclear RNA was used as an internal normalized reference, and fold changes were calculated by relative quantification ($2^{-\triangle\triangle Ct}$). To examine the specificity of the qRT-PCR, the dissociation curve analysis was performed after a completed PCR. All samples were amplified in triplicate and the data analysis was carried out using the MxPro qPCR system software (Stratagene, USA).

Transfection

miR-27a mimic, miR-27a negative control (miR-NC), miR-27a inhibitor were synthesized and purified by RiboBio. RNA oligonucleotides were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) and medium was replaced 6 hours after transfection according to the manufacturer's recommendations. A final concentration of 50 nM was used. After 24h or 48h transfection, cells were used for subsequent experiments including proliferation, apoptosis, migration, and invasion assays.

Cell proliferation assay

Twenty-four hours after transfection, cells were placed in 96-well plates (1000/well) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 4 days. Proliferation of cancer cells was measured per 24h by the MTT (3-2, 5-diphenyl tetrazolium bromide) assay (Sigma, San Francisco, CA, USA).

Cell cycle assay

After 48h transfection in 6-well plates, the cells were harvested and washed twice with cold PBS. Cells were fixed in 75% ethanol at 4°C overnight. Staining for DNA content was performed with 50 ug/ml propidium iodide and 50 ug /ml ribonuclease A at 4°C for 30 min in the dark. Populations in G0 /G1, S and G2/M phase were were analysed by FACS (BD, Bedford, MA, USA). Data were collected and analyzed with the CELLQuest and ModFit LT software.

Migration and invasion assay

The migration and invasive potential of cells was measured in a 24-well transwell plate with 8 mm pore polycarbonate membrane inserts, according to the manufacturer's protocol (Corning, New York, USA). For transwell migration assay, 3×10^4 cells for HepG2 or 5×10^4 cells in 200 ul serum free medium containing 0.1% BSA were plated in the upper chamber. For invasion assay, chamber inserts were coated with the matrigel (200 ng/ml) (BD Biosciences, Bedford, MA, USA). Then, 5×10^4 cells for HepG2 or 7×10^4 were plated in the upper chamber. The lower chambers were filled with 0.6 ml of DMEM medium containing 10% FBS. After incubation for 24 h at 37 °C in a humidified incubator with 5% CO_2, the upper chambers were wiped with cotton wool to remove the non-migratory or non-invasive cells. The invasive cells attached to the lower surface of the membrane insert were fixed in 100% methanol at room temperature for 10 min, airdried, stained with 0.05% crystal violet, and counted by photographing the member through the microscope (×200 magnifications).

Statistical analysis

Data are presented as mean±SD. Data were analyzed using the Mann–Whitney U test for continuous variables and the Student's t-test for normally distributed variables.
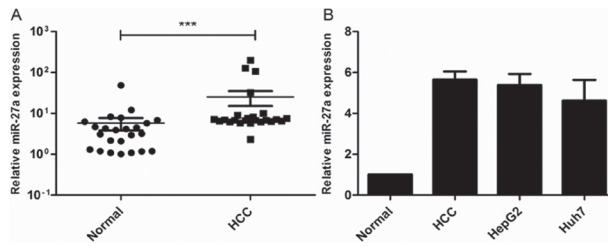
Figure 1. miR-27a Expression is Upregulated in HCC Tissues and Cells Lines. (A) qRT-PCR analysis of miR-27a in 24 pairs of HCC tissues and adjacent nontumorous tissues (Normal). (B) miR-27a expression in the normal, HCC tissues and HCC cell lines HepG2 and Huh7. ***$p<0.001$
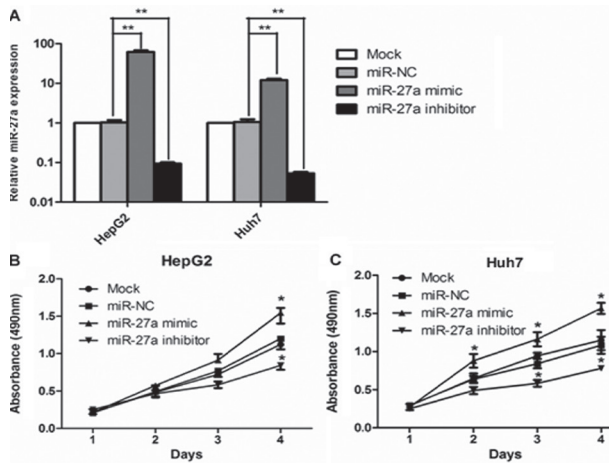

Figure 2. Transient Transfection of miR-27a Mimic (or inhibitor) Promotes (or inhibits) the Cell Proliferation of HCC Cell Lines. (A) The expression of miR-27a in HepG2 and Huh7 cells with or without transfection of miR-negative control (miR-NC), miR-27a mimic and inhibitor were confirmed by qRT-PCR. (B) The effect of miR-27a on cell proliferation of HCC cell lines was analysed by MTT assay. Data are shown as mean±SD from three independent experiments. *$p<0.05$ versus miR-NC

Values of $P<0.05$ were accepted as statistically significant.

Results

miR-27a Is Significantly Up-regulated in HCC Tissue Samples and HCC Cell Lines

The expression of miR-27a in tissues of HCC patients has not been well documented. To test whether miR-27a was correlated with HCC development, we measured the expressions of miR-27a in HCC tumors and adjacent non-tumor tissues using quantitative RT-PCR. The results showed that the expression of miR-27a in cancer tissues was significantly higher than those in adjacent non-tumor tissues (Figure 1, $p<0.01$, Mann–Whitney). We also found that miR-27a was highly expressed in HepG2 and Huh7 cells. These findings indicated that the overexpression of miR-27a might be associated with hepatocarcinogenesis.

miR-27a Promotes Cell Proliferation By Decreasing The Percentage of Cells in G1/G0 Phase

The significant overexpression of miR-27a in HCC tissues indicated that it possibly functions as an oncogene. We examined the effects of miR-27a on cell proliferation in HepG2 and Huh7 cells transfected with or not, miR-NC,
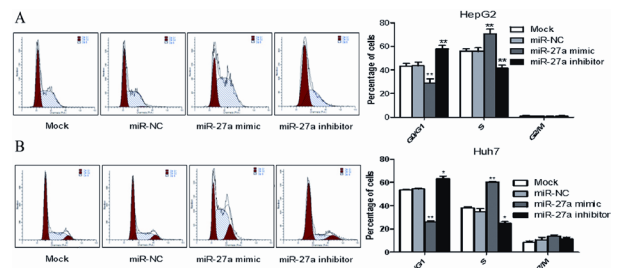

Figure 3. Effect of miR-27a on HCC Cell Cycle, Migration and Invasion. A, The effect of miR-27a on HepG2 or Huh7 cell cycle distribution was analysed by flow cytometry. Data are shown as mean±SD from three independent experiments. *$p<0.05$; **$p<0.01$ versus miR-NC

miR-27a mimic or miR-27a inhibitor. The quantitative real-time PCR analysis displayed that the expression of miR-27a was increased approximately 62-folds (HepG2) and 12-folds (Huh7) in cells transfected with 50nM miR-27a mimic but decreased 11-folds (HepG2) and 20-folds (Huh7) with 50nM miR-27a inhibitor (Figure 2A). A significant increase in cell proliferation was observed over time in both cells lines transfected with miR-27a mimic compared with miR-NC or non-transfected cells (Figure 2B and 2C). In contrast, the viability of cells transfected with miR-27a inhibitor significantly decreased (Figure 2B and 2C), indicating that miR-27a could enhance HCC cell proliferation.

To investigate the mechanism underlying the miR-27a mediated cell proliferation, we analyzed cell cycle distribution by flow cytometry. Our data showed the percentages of miR-27a mimic transfected HepG2 and Huh7 cells in the G0/G1 phase were 15% (HepG2) and 28% (Huh7) lower than that of miR-NC or non-transfected cells, while in the S phase 16% (HepG2) and 24% (Huh7) higher (Figure 3). The percentages of cells transfected with miR-27a inhibitor in the G0/G1 phase were 15% (HepG2) and 10% (Huh7) more than that of miR-NC or non-transfected cells, while in the S phase 14% (HepG2) and 11% (Huh7) less (Figure 3). Taken together, the results suggest that miR-27a could enhance HepG2 and Huh7 cell proliferation by decreasing the percentage of cells in G1/G0 Phase.

miR-27a Positively Regulating Cell Migration and Invasion

We further investigated whether miR-27a contributed to cell migration and invasion properties. HepG2 or Huh7 cells transfected with or not, miR-NC, miR-27a mimic or miR-27a inhibitor, were applied to transwell assays. We found that miR-27a mimic increase the relative migration in HepG2 and Huh7 cells by 77% and 150% ($p<0.01$), respectively (Figure 4). Meanwhile, cell invasion was markedly promoted in both two cell lines transfected with miR-27a mimic, exhibiting a 112% and 144% increase of invasion with a statistcally significant difference ($p<0.01$) in the HepG2 and Huh7cells, respectively (Figure 4). In contrast, cells transfected with miR-27a inhibitor significantly decreased the migration and invasion ability ($P<0.01$) (Figure 4). These results suggested that miR-27a function can promote migration and invasion in HCC cells.

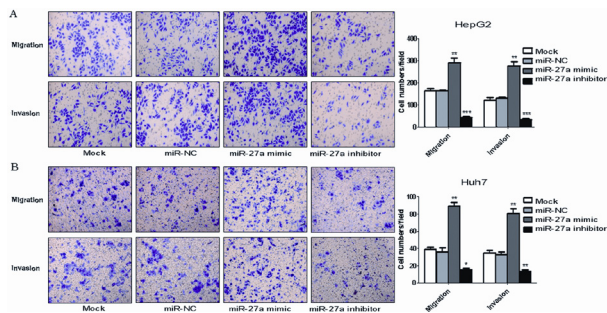We also tested if miR-27a could affect cancer cell

Figure 4. Effect of miR-27a on HCC Migration and Invasion. Tanswell assay was employed to evaluated the effect of miR-27a on the migration and invasion ability of HCC cell. The invasive cells were photographed (images) and counted in 5 random fields per well at 200 × magnification. Data are shown as mean±SD from three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ versus miR-NC

apoptosis by overexpression or underexpression. The results demonstrated that altered miR-27a expression neither increased nor decreased cell apoptosis compared to control cells (data not shown). In summary, our data indicates that miR-27a can increase HCC cell proliferation, migration, and invasion, but has no obvious effect on cell apoptosis.

Discussion

In this study, we examined the expression levels of miR-27a in HCC and adjacent non-tumor samples. qRT-PCR showed that miR-27a was significantly up-regulated in HCC samples. Meanwhile, we also found that miR-27a was highly expressed in HepG2 and Huh7 cells. To investigate the function of miR-27a in HCC, we assessed the effect of it on HepG2 and Huh7 cells by both overexpression and downregulation experiments. Overexpression of miR-27a promoted cell proliferation, G1/S cell cycle transition, migration and invasion in HCC cells. Conversely, inhibition of miR-27a reduced HCC cell proliferation, migration and invasion, and delayed the G1/S transition. Collectively, these findings suggest that upregulation of miR-27a may promote the initiation and progression of HCC.

Until now, DNA, RNA or protein profile of cancer research has not fully elucidated the pathogenesis of HCC. One of the most modern and progressive approaches for molecular characterization of tumors today is based on miRNA expression profiles. Aberrant expression of miRNA is associated with the pathogenesis of a variety of cancers, including HCC. Several studies showed that miRNA expression profiles between HCC and non-tumor tissue are significantly different (Kutay et al., 2006; Budhu et al., 2008; Jiang et al., 2008; Ladeiro et al., 2008; Ji et al., 2009). Aberrant miRNA expression in liver tissues or serum samples could be used as diagnostic or prognostic marker in HCC (Jiang et al., 2008; Li et al., 2008; Ji et al., 2009; Li et al., 2010). Recently, Zhou et al. (Zhou et al., 2011) combined seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) in plasma as a diagnostic panel with a better diagnostic performance to differentiate HCC from control groups of healthy, chronic hepatitis B, and liver cirrhosis. Low

expression level of miR-27a in plasma was observed in patients with HCC compared with control groups of healthy, chronic hepatitis B, and liver cirrhosis (Zhou et al., 2011). However, we found that miR-27a was significantly up-regulated in HCC samples. miR-27a has been found to be an oncogene, which is abnormally up-regulated in several types of cancers, including gastric cancer (Liu et al., 2009), colon cancer (Chintharlapalli et al., 2009), pancreatic cancer (Szafranska et al., 2007; Ma et al., 2010), breast cancer (Guttilla et al., 2009), ovarian cancer (Li et al., 2010), and kidney cancer (Gottardo et al., 2007), pediatric acute lymphoblastic leukemia (ALL) (Lerner et al., 2011). It has been demonstrated that miR-27a play important roles in mediating cancer cell proliferation, cell cycle, apoptosis, migration and drug resistance (Mertens-Talcott et al., 2007; Liu et al., 2009; Ma et al., 2010; Lerner et al., 2011). Consistently, we here showed that inhition of miR-27a suppressed proliferation, migration and invasion of both HepG2 and Huh7 cell lines, indicating the oncogenic role of miR-27a in HCC cells. Several studies have identified prohibitin (Liu et al., 2009), ZBTB10 (Mertens-Talcott et al., 2007), Myt1 (Mertens-Talcott et al., 2007), FOXO1 (Guttilla et al., 2009), Sprouty2 (Ma et al., 2010), FBW7 (Lerner et al., 2011) as miR-27a target genes.

In conclusion, our study demonstrated that miR-27a was significantly up-regulated in HCC tissues, HepG2 and Huh7 HCC cell lines. miR-27a is a potential novel oncogenic miRNA in HCC and might play a part in enhancing cell proliferation, migration and invasion of HCC cells. These results indicates that inhibition of miR-27a may be a potential therapeutic strategy for the treatment of HCC. It is worthy to study fully understand the molecular mechanism by which miR-27a plays a role as oncogenic genes in the development and progression of HCC.

Acknowledgements

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