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Original Article

The negative feedback loop of miR-122 and c-myc in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is a highly malignant hepatoma and the third most common cause of cancer-related death in the world. Decreased miR-122 expression wherase elevated c-Myc expression were observed and associated with HCC development and poor prognosis. In this study, we demonstrated the negative correlation between c-Myc and miR-122 in clinical HCC samples, hepatoma cells and dimethylnitrosamine (DEN)-induced HCC rat model, suggesting that a potential mechanism of hepatocarcinogenesis and a promising strategy for HCC treatment and diagnosis.

Keywords: miR-122, c-Myc, tumorigenesis, HCC

Introduction

MicroRNAs (miRNA) are a class of evolutionally conserved non-coding small RNAs, binding 3'untranslated regions (3'UTR) of target genes and post-transcriptionally repressing the gene expression [1]. Several key observations underscore gene expression can be regulated by miRNA through binding the gene coding regions or 5'UTR [2-4].

MiR-122, a live-specific miRNA, accounts for 70% of the total miRNA population in live and has attributed to regulate hepatocyte development, cell differentiation, lipid metabolism and virus replication [5-7]. Moreover, miR-122 can repress hepatocellular carcinoma (HCC) development due to regulate the target genes involved in cell migration, proliferation, differentiation, apotosis and angiogenesis propertites [1, 5, 8]. As a highly malignant hepatoma, hepatocarcinogenesis is a stepwise process with multiple genes altered [9]. Subsequent functional studies further showed that reduction of miR-122 suggested a poor prognosis and diagnosis in patitents with hepatitis B virus (HBV) infection and human HCC [1, 10]. In addition, dysregulation of miR-122 can lead to HCC spontaneously with age in miR-122 knockout mice and surprisingly miR-122 restoration showed significantly reduction of HCC development, indicating the important physiological role and intrinsic tumor suppressor function of miR-122 [11, 12]. Furthermore, miR-122 served as a serum biomarker in early liver-related diseases, also has the abilities to sensitize HCC cancer cells to the treatment of radiotherapy and chemotherapy [13-16].

The c-Myc protein encoded by the proto-oncogene Myc was frequently up-regulated in various tumors [17, 18]. As a transcription factor, dimer of c-Myc and Max can regulate the promoter activities of target genes involved in cell growth and proliferation. Moreover, c-Myc activation can initiate hepatocarcinogenesis in several c-Myc transgenic animal models such as Tet-o-Myc [19-21].

The recent studies had showed that c-Myc had the abilities to repress several miRNA expression in HCC such as miR-17-5p, let-7a, miR-26a as well as miR-122 [22-24]. Similarly, reciprocal regulation of c-myc and miR-122 was explored [19]. Considering the fundamental roles of miR-122 and c-Myc in hepatocarcinogenesis, we further explored the feedback loop between c-Myc and miR-122 in HCC patients, hepatoma cells and Dimethylnitrosamine (DEN)-induced HCC rat model [25].

Table 1. primers used for real-time PCR

Human primers used for real-time PCR	
h-c-myc-F	5'-CCAACAGGAACTATGACCTC-3'
h-c-myc-R	5'-CTCGGTCACCATCTCCAGCT-3'
h-GAPDH-F	5'-GGTGAAGGTCGGTGTGAACG-3'
h-GAPDH-R	5'-CTCGCTCCTGGAAGATGGTG-3'
Rats primers used for real-time PCR	
c-myc-F	5'-GTCCTCAAGAGGTGCCATGT-3'
c-myc-R	5'-CTCGCCGTTTCCTCAGTAAG-3'
GAPDH-F	5'-GGCAAGTTCAACGGCACAGT-3'
GAPDH-R	5'-TGGTGAAGACGCCAGTAGACTC-3'

Material and methods

Human Specimens

Liver sections from 15 HBV-infected HCC patients and corresponding adjacent normal tissues were collected for miR-122 and c-Myc mRNA detection. Standards for diagnosis of HCC had been shown before [17]. All the HCC patients participanted were hospitalized in Beijing You'an hospital of Captial University of Medical Sciences from 2010 to 2012 and provided the informed consent in written form. The study protocols were approved by the Ethics Committee of Beijing You'an hospital.

Reagents and antibodies

The chemically synthesized miR-122 mimic and non-specific control, the miR-122 inhibitor and non-specific control were purchased from RiboBio Co., Ltd. (Guangzhou, China). Superscript™ RT reagent kit (DRR037A) was purchased from Takara BioInc., Shiga, Japan; Trizol reagent and RNase-free DNAse I were bought from Invitrogen; the mouse anti-c-myc (sc-40), the mouse anti-human actin and GAPDH were bought from Santa Cruz Biotechnology; the horseradish peroxidase-conjugated secondary antibodies and the ECL-Plus chemiluminescence system were purchased from Zhongshan Goldenbridge Biotechnology (China) and Applygen Technologies (Beijing, China), respectively.

Cell culture and transfection

Huh-7 and HepG2 as the human hepatoma cell lines were obtained from the ATCC (Manassas, VA, USA). For transfection studies, Huh-7 and HepG2 cells were planted in six-well plates 12~18 h before transfection. Then, cells were washed with 2 ml RNA-free Opti-MEM (Invitrogen) and 1.5 ml RNA-free Opti-MEM was added in each six-well plates at least 1 h before tansfection to improve transfection efficiency. Then, 50 nM miR-122 inhibitor or mimic was transfected using Lipofectamine 2000 (Invitrogen). Each treatment was repeated at least three times.

Animal studies

According to the "guide for the Care and Use of laboratory Animals" (NIH publications No.80-23, revised 1996) by the National Academy of Sciences, Twelve Pathogen-free male Sprague Dawley (SD) rats (weighing 160-180 g) were purchased from Institute of Laboratory Animal Science and divided into two groups randomly. To build live cancer models in rats, two groups were chosen and injected DEN at the dose of 70 mg/kg or PBS as control by weekly intraperitoneal (i.p.) for 20 weeks in total. According to ether anesthesia, rats were sacrificed after the last DEN injection and lives of the rats were removed and kept in -80°C immediately after sacrifice.

Western-blot analysis

An equal amount of protein from cell lysates or rat lives was separated by a 10% SDS-polyacrylamide gel and probed with an anti-c-myc, an anti-actin or GAPDH antibodies. Next, the film was incubated with the appropriate HRP-conjugated secondary antibodies and visualized using an ECL detection kit .

RNA isolation and real-time PCR

Total RNA was extracted with the Trizol Reagent from treated cells or rat lives and treated with RNase-free DNAse I to rule out DNA interference. Then, as recommended by the manufacturer, RNA was reverse transcribed using the Superscript™ RT reagent kit (Takara). Specific primers used to detect c-myc and GAPDH mRNA by real-time PCR was listed in **Table 1**.

Real-time fluorescence quantitative PCR (RT-PCR) was performed using the SYBR Green Premix Reagent (Takara BioInc., Shiga, Japan). Two steps were used in PCR cycling, and the conditions were as follows: 95°C 30 s, followed by 95°C 15 s and 60°C 45 s for 40 cycles. The specificities of primers were estimated by Melt-

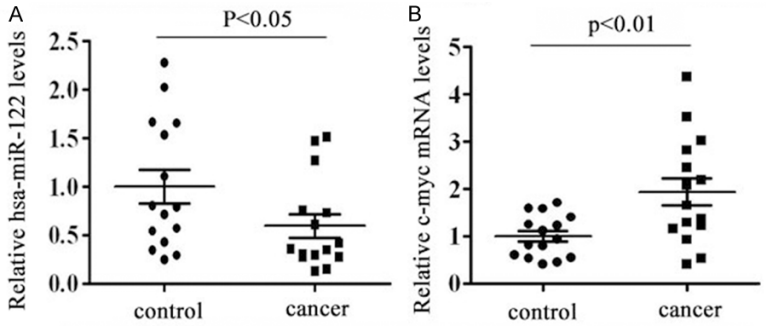

Figure 1. Changes of miR-122 and c-myc mRNA levels in lives of HBV-infected HCC patients. A and B: miR-122 and c-myc mRNA levels were detected by real-time PCR in livers of HCC compared to adjacent normal livers.

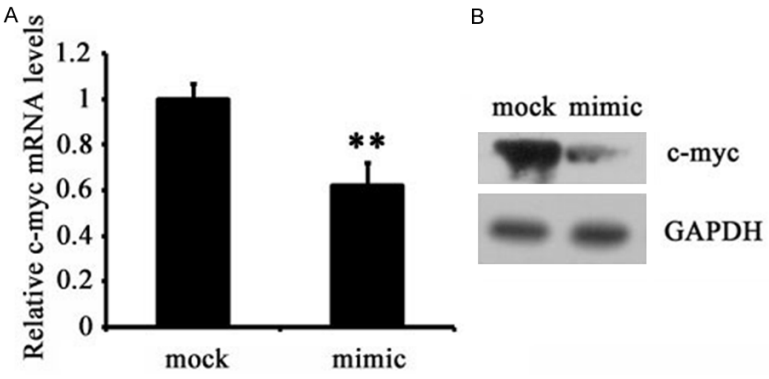

Figure 2. Expression of c-myc mRNA and protein levels was down-regulated by miR-122 in HepG2 cells. A and B: HepG2 cells were transfected with 50 nM miR-122 mimic or negative control. At 48 h after transfection, c-myc mRNA and protein levels were analyzed by RT-PCR and western-blot, respectively.

curve analysis. Each experiment was performed in triplicate.

TaqMan miRNA analysis

Mature miR-122 levels in rat livers were detected by TaqMan miRNA analysis Kit purchased from Applied Biosystems, USA, and U6 was used for endogenous control. MiR-122 levels were performed by RT-PCR according to the manufacturer recommended.

Statistical analysis

All the RT-PCR experiments were performed in triplicate, and all experiments used in the study were repeated three times. The results are presented as the means ± standard deviation. Comparison of two groups used Student's *t*-test and *P* value < 0.05 or P < 0.01 considered as significant differences.

Results

Suppression of miR-122 whereas over-production of c-Myc in HBV-infected HCC patients

To demonstrate the abnormal expression of miR-122 and c-Myc in HCC patients, we detected the two gene expression in HCC tissue compared to adjacent normal tissue from 15 HCC patients by real-time PCR. As shown in **Figure 1A**, mature miR-122 expression exhibited markedly decreased by about 40% in HCC tissue (P < 0.05). Considering the oncogene c-Myc plays important roles in promoting tumorigenesis, c-Myc expression was also examined by real-time PCR. Notably, c-Myc mRNA was significantly increased by about one time in HCC tissue (P < 0.01). The data had shown that miR-122 suppression wherase elevated c-Myc expression happened in clinical HCC samples.

miR-122 suppressed c-Myc expression in human hepatoma cell lines

Next, to further explore the correlation of miR-122 and c-Myc expression in human hepatoma cell lines, HepG2 cells with miR-122 lower expression were transfected with 50 nM miR-122 mimic or negative control. Then, c-Myc mRNA and protein levels were detected by real-time PCR and western-blot, respectively. As can be seen in **Figure 2A** and **2B**, c-Myc mRNA was significantly decreased by 38% with miR-122 mimic transfection. Similarly, c-Myc protein levels were dramatic decreased.

Furthermore, Huh7 cells were selected to elucidate whether c-Myc was subject to regulation by miR-122 because this is the only human hepatoma cell line with constitutive expression of miR-122. Huh7 cells were transfected with 50 nM miR-122 inhibitor or negative control. Next, we detected c-Myc expression by RT-PCR

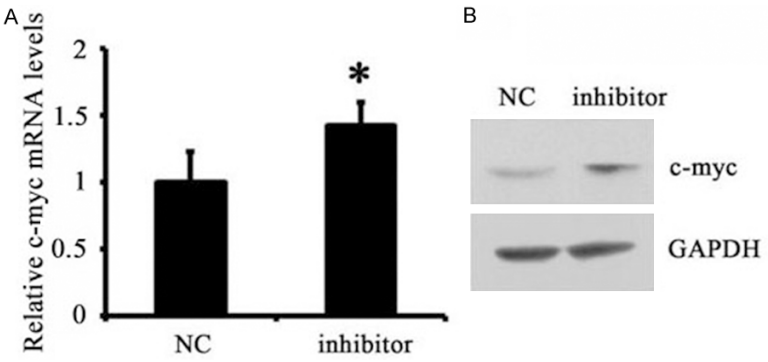

Figure 3. miR-122 knockdown in Huh7 cells induced up-regulation of c-myc. A and B: Huh7 cells were transfected with 50 nM miR-122 inhibitor or negative control. c-myc mRNA and protein levels were performed by RT-PCR and western-blot at 48 h after transfection.

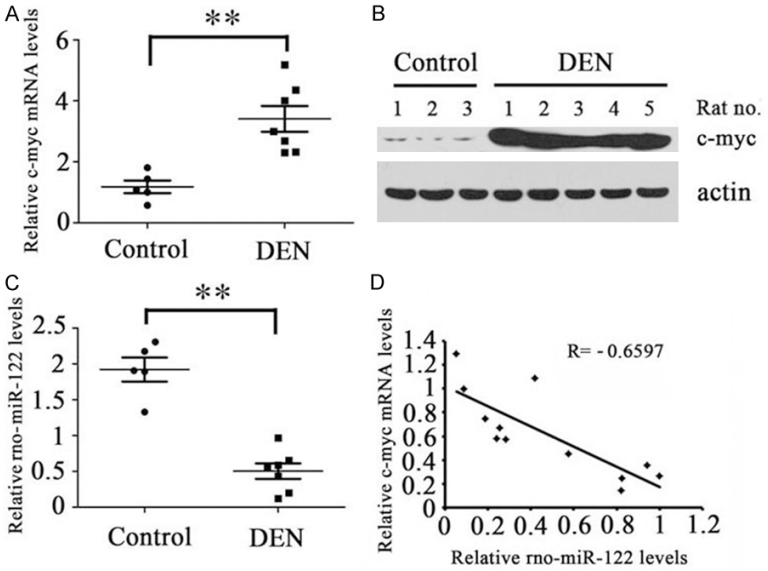

Figure 4. Changes of miR-122 and c-myc expression in DEN-induced HCC rats. A and B: c-myc mRNA and protein levels in lives of DEN-induced HCC or PBS control groups were analyzed by RT-PCR and western-blot, respectively. C: miR-122 levels in lives of DEN-induced HCC or PBS group were also detected by RT-PCR. D: Correlation of miR-122 and c-myc mRNA levels were analyzed in lives of DEN-induced HCC rat model.

and western-blot, respectively. As demonstrated in **Figure 3A** and **3B**, c-Myc mRNA was dramatically elevated by 42% synchronized with the increase of c-Myc protein levels. Taken together, the data showed that miR-122 can repress c-Myc expression in HepG2 and Huh7 cells.

The negative correlation between miR-122 and c-Myc in DEN-induced HCC rats

To assess the correlation between c-Myc and miR-122, we utilized the pathogen-free male SD rats (6~8 weeks, weighing 160-180 g) to build the hepatocarcinogenesis model induced by DEN. The rats need about 20 times intraperitoneal injection of DEN (70 mg/kg) weekly to cause HCC. After 20 weeks injection, rats were sacrificed. Compared to untreated rats with PBS injection, DEN-treated rats exhibited obviously higher c-Myc mRNA and protein expression whereas lower miR-122 levels in live tissues 20 weeks after first DEN injection (**Figure 4A-C**). As expected, decreased miR-122 wherase elevated c-Myc expression displayed a negative correlation in livers of DEN-treated rats ($r = 0.6597$, $P < 0.05$) (**Figure 4D**).

To systematically elucidate the regulators and miR-122 target genes relevant to HCC and the reciprocal regulation between miR-122 and c-Myc in liver, we proposed a model based on the data we obtained and previous studies [1, 10, 17, 19, 26] (**Figure 5**).

Discussion

It's well known that miR-122 acts as a tumor suppressor in the liver and its down-regulation is a characteristic of HCCs with poor prognosis [27, 28]. Previous studies have illustrated that exogenic miR-122 introduced can inhibit the development of HCC [11]. Additionally, oncogene c-Myc whose overexpression will induce spontaneous tumors resembling HCC is down-regulated by miRNA such as miR-34a, let 7, miR-145 and miR-122 [19, 29, 30]. Furthermore, the underlying reciprocal mechanism of miR-122 repressed by c-Myc was reported by directly binding miR-122 promotor region and down-regulating miR-122 transcription factor HNF3β whereas miR-122 can induce c-Myc suppres-

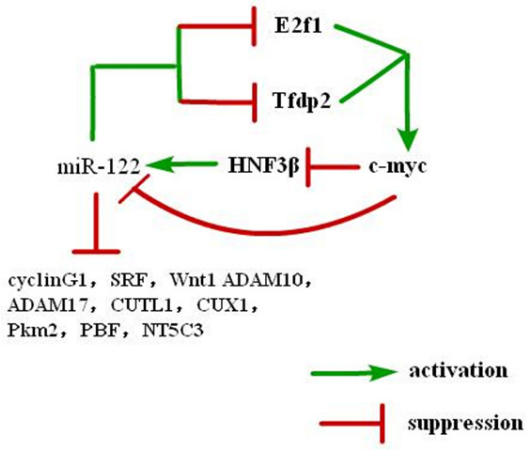

Figure 5. The model of the regulators and target genes relevant to HCC of miR-122 and the reciprocal regulation between miR-122 and c-Myc in liver.

sion by indirectly targeting the transcription factor E2f1 and Tfdp2 in hepatoma cells and mice models [19]. Since c-Myc was increased in most tumors resembling HCC and played critical roles in modulating HCC development, thus, to clarify the regulation mechanism between c-Myc and miR-122 and dicuss the perspectives of miR-122 based therapy is essential for early diagnosis and good prognosis of HCC.

In this study, we verified the miR-122 downregulation wherase increased c-Myc expression in clinical HCC samples. Next, reduced c-Myc expression was validated by miR-122 transfection in HepG2 cells and miR-122 knockdown in Huh7 cells resulted in downregulation of c-Myc highlighting that c-Myc expression was regulated by miR-122 in hepatoma cells. Eventually, we demonstrated the negative correlation between c-Myc and miR-122 in DEN-induced HCC rat model.

The negative feedback loop between c-Myc and miR-122 was confirmed in human clinical samples, human hepatoma cells and DEN induced HCC rat model, which may provide a new promising strategy for HCC diagnosis and treatment.

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Disclosure of conflict of interest

None.

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