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Original article

MicroRNA-22 is down-regulated in hepatitis B virus-related hepatocellular carcinoma

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ABSTRACT

MicroRNAs (miRNAs) are a new class of short noncoding RNAs with post-transcriptional regulation and participate in diverse physiological and pathological processes. MiR-22, ubiquitously expressed in various tissues, plays a functional important role in life processes and is recently proved to involve in many cancers. In present study, we had shown that miR-22 was down-regulated much more obviously in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) cell lines as well as in clinical tissues. Cyclin-dependent kinase inhibitor 1A (CDKN1A) was found to be inversely correlated with miR-22 and was identified as a target of miR-22. Overexpression of miR-22 in vitro effectively suppressed CDKN1A expression and inhibited cell proliferation. We conclude that miR-29c may play an important role as a tumor suppressive microRNA in the development and progression of HBV-related HCC by targeting CDKN1A.

1. Introduction

Hepatocellular carcinoma (HCC) is the third aggressive cancers in the world and the second in China [1]. It has demonstrated that hepatitis B virus (HBV) infection is one of the key risk factors for HCC [2], which is greatly detrimental to human health and affects the quality of people's life [3]. China, a country of high prevalence of HBV infection, has about 12 million chronic HBV carriers and approximately 30 million chronic hepatitis B patients. Furthermore, the number increasing with a rate of $(10 \sim 25) \times 10^5$ cases every year [4]. Therefore, it is of great importance to investigate the pathogenesis of HBV infection as well as the carcinogenesis of HBV-related HCC. The discovery of miRNAs brings new possibility for devising new strategies for its prevention and therapy.

MicroRNAs (miRNAs) are a new class of small noncoding RNAs that may bind to the imperfect complementary sequences of their target mRNAs at the 3′ untranslated regions (3′-UTR) and induce mRNA degradation or translational repression [5]. MiRNAs have been shown to regulate gene expression in complicated physiological and pathological processes [6], such as development, proliferation, apoptosis, stress responses and so on [7]. Recent studies have found that aberrant miRNA expression is closely associated with a number of disease and cancer [8,9]. Moreover, a large number of studies have proved that miRNA expression is nearly ubiquitous deregulation in different cancer cells [10,11]. In addition, a growing number of studies has documented that altered expression of specific miRNAs contribute to tumorigenesis and miRNAs may act as tumor suppressors or oncogenes [12–14].

MiR-22 was originally detected in HeLa cells, but was later found to be ubiquitously expressed in various tissues [15]. The gene encoding miR-22 is found on the short arm of chromosome 17, and is highly conserved across many vertebrate species, including chimp, mouse, rat, dog and horse. This level of conservation suggests that miR-22 play a functional important role in life processes. Recently, more and more studies have found that miR-22 was down-regulated in many cancers, including cholangiocarcinoma, multiple myeloma and hepatocellular carcinoma [16]. In HBV-related HCC, the role of miR-22 remains largely unknown.

Here, we investigated whether miR-22 could have a role in HBV-related HCC. We found that miR-22 was s expressed relatively weakly in HBV-related HCC cell lines and in HBV clinical specimens compared with their corresponding controls. CDKN1A, as a target gene of miR-22, was found to be inversely correlated with miR-22 levels both in cell lines and in clinical specimens. We also moreover demonstrated that the overexpression of miR-22 in HepG2.2.15 cells effectively suppressed CDKN1A expression and cell proliferation. In this study, we report that miR-22 regulates cell proliferation by targeting CDKN1A in HBV-related cell lines, showing that miR-22, as a tumor suppressor miRNA, may play an important role in the development of HBV-related HCC.

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2. Materials and methods

2.1. Clinical samples

Paired surgical specimens were obtained with documented informed consent from patients in affiliated Hospital of Jining medical University, China, which included one normal hepatic tissue as well as 30 pairs of tumors and adjacent non-cancerous tissues (NT). The patients had been diagnosed with HBV-related HCC. All tissues were frozen in liquid nitrogen after surgical resection and stored at −80 °C.

2.2. Cell culture

HepG2 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, China) containing with 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/mL penicillin and streptomycin. HepG2.2.15 cell lines (purchased from ATCC) which were transfected with full-length HBV genome was cultured in DMEM (Hyclone, China) with 10% FBS and 350 mg/L antibiotic G-418 sulfate (Promega, USA). All cells were cultured at 37 °C in the atmosphere of 5% CO2.

2.3. MiR-22 expression analysis by real-time quantitative PCR

qRT-PCR was performed to detect the expression level of miR-22 in cell lines and clinical specimens. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription using an NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen). All-in-one miRNA qPCR Primer (GeneCopoeia) was used as Primers to amplify miRNA-22. The expression levels of snRNA U6 were assayed to normalize the relative abundance of miR-22 by $2-\Delta\Delta CT$ method. The PCR reactions were performed using a PCR Lightcycler 480 (Roche) with the EXPRESS SYBR GreenER™ miRNA qRT-PCR Kit (TaKaRa). After denaturation 2 min at 95 °C, 40 amplification cycles were performed as following: denaturation at 95 °C for 5 s, annealing at 60 °C for 31 s. Fluorescence was collected automatically during amplification, and the melting curve for the product was obtained. All reactions were performed in triplicate.

2.4. MiR-22 target predictions

Bioinformatic method was using to predict the putative target genes of miR-22. We select three common algorithms, including TargetScan (http://www.targetscan.org/), Pictar (http://pictar.bio.nyu.edu/) and miRanda (http://microrna.sanger.ac.uk/). CDKN1A, which was predicted in three algorithms, was selected as a target of miR-22. We also used the TargetScanS 5.1 algorithm for further genome-wide miRNA target prediction. RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) was used to predict the duplex between miR-22 and the 3'-UTR of CDKN1A,

2.5. Real-time PCR

The expression levels of CDKN1A were measured by RT-PCR in cell lines and clinical specimens. Total RNA was isolated as described above. cDNA was synthesis using the First Strand cDNA Synthesis kit ReverTra Ace-α- (TOYOBO). The PCR reaction was performed in a single reaction of 20 μL volume. Primers for the amplification of the CDKN1A, gene were as follows: forward primer: 5′-gacaccactggagggtgact-3′, reverse primer: 5′-caggtccacatggtcttcct-3′. The human GADPH gene was used as an endogenous control and amplified with the following primer pairs: forward primer: 5′-AGCCACATCGCTCAGACAC-3′, reverse primer: 5′-GCCCAATACGACCAAATCC-′.

PCR was conducted as follows: 94 °C for 3 min, followed by 32 cycles of 94 °C for 30 s, 55 °C for 40 s and 72 °C for 40 s. The products were analyzed on 1.2% agarose gel that was stained with ethidium bromide.

2.6. Reporter plasmid construction and luciferase reporter assay

To create a luciferase reporter construct, 3′-UTR segments of CDKN1A, that contained the putative binding sites for miR-22 were synthesized by PCR using the following primers: forward: 5′-tggtacccccagctacccttcttctcc-3′ (KpnI); reverse: 5′-ggagatctgccagtgtctccctcctaga-3′ (BglII). After digestion and purification, the products were cloned into the KpnI and BglII sites on the pGL3-control (Promega, Madison, WI) vector downstream of the luciferase gene to generate the pGL3-CDKN1A, construct.

HepG2 cells were seeded on a 24-well plate at a density of 2×10^5 cells per well. After 24 hours, cells were co-transfected with miR-22 and either pGL3-CDKN1A or pGL3-control using lipofectamine 2000. The samples were also co-transfected with 50 ng of pRL-TK plasmid expressing Renilla luciferase to determine the transfection efficiency. Twenty-four hours after transfection, all cells were harvested, and Firefly and Renilla luciferase activity were measured with the Dual-Luciferase Reporter system (Promega). Firefly luciferase activities were standardized to the Renilla luciferase activities as an internal standard of transfection efficiency. All experiments were carried out with triplicate samples.

2.7. Transfection

One day before transfection, HepG2.2.15 cells were trypsinized and plated in 6-well or 96-well. Transfection was performed with 2 μg of either miR-22 or negative control using lipofectamine 2000 according to the manufacturer's instructions. Four to six hours after transfection, the cells were replenished with fresh medium containing FBS.

2.8. Hepatitis B surface antigen and hepatitis B e antigen assay

To observe the effect of miRNA-22 on HBV at the protein level, we measured the viral proteins of HBsAg and HBeAg in the culture supernatant from the transfected cells. Forty-eight hours after transfection of miR-22 or negative control, the supernatant was collected and carried out using ELISA kit (Autobio, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm using Bio-Rad protein assay (USA), and the assays were performed in triplicate. Inhibitory rates were calculated according to the following: inhibitory rate (%) = (C control-C tested)/C control × 100%.

2.9. Cell proliferation assay

To asses the effect of miRNA-22 on cell viability, HepG2.2.15 cells were seeded on 96-well plate and transfected 24 hours later with either miR-22 or negative control using lipofectamine 2000. At different time points (0, 24, 48 and 72 hours), the culture media was removed and was replaced with culture medium containing 10 μL of sterile CCK8 (Dojindo, Kumamoto, Japan) for each well. The cells were then incubated for 1 hour at 37 °C, and the absorbance of the suspension was measured at 450 nm using Bio-Rad protein assay.

2.10. Quantitative PCR

HepG2.2.15 cells were transfected with either miR-22 or negative control in a 6-well plate. The cells, which were not transfected were control. After 48 hours, RNA was extracted and