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miR-29c targets TNFAIP3, inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma

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ABSTRACT

Recent studies have revealed that microRNA-29c (miR-29c) is involved in a variety of biological processes including carcinogenesis. Here, we report that miR-29c was significantly downregulated in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) cell lines as well as in clinical tissues compared with their corresponding controls. Tumor necrosis factor alpha-induced protein 3 (TNFAIP3), a key regulator in inflammation and immunity, was found to be inversely correlated with miR-29c levels and was identified as a target of miR-29c. Overexpression of miR-29c in HepG2.2.15 cells effectively suppressed TNFAIP3 expression and HBV DNA replication as well as inhibited cell proliferation and induced apoptosis. 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 TNFAIP3. Thus miR-29c and TNFAIP3 represent key diagnostic markers and potential therapeutic targets for the prevention and treatment of HBV infection.

1. Introduction

microRNAs (miRNAs) are a class of non-coding, single-stranded RNA molecules containing 19–25 nucleotides (nt), which are cleaved from a 70- to 80-nt partially duplexed precursor (pre-miRNA). Although the precise mechanism how miRNAs regulate gene expression is not fully understood, several experiments have shown that most miRNAs play important roles at the post-transcriptional level by imperfect base-pairing with 3′-untranslated regions (3′-UTR) of target mRNAs [1]. miRNAs were found to be key regulators in a variety of biological processes, including cell cycle, cell differentiation, proliferation and apoptosis [2,3]. A growing amount of evidence has indicated that miRNAs are extensively involved in the pathogenesis of many different human cancers [4]. The importance of miRNAs in tumorigenesis is highlighted by the fact that 50% of the genes that encode miRNAs reside in cancer associated genomic regions or fragile sites [5].

Hepatocellular carcinoma (HCC) has the third highest mortality rate among cancers worldwide and is second highest in China [6]. Epidemiological studies have demonstrated that hepatitis B virus (HBV) infection is one of the key risk factors for HCC [7]. Furthermore, although the incidence of HBV infection is increasing, the available therapy is only partially effective against the virus. Therefore, investigating the pathogenesis of HBV infection as well as the carcinogenesis of HBV-related HCC is important. The discovery of miRNAs may provide a valuable new approach for devising new strategies for its prevention and therapy.

The miR-29 family members (miR-29a, miR-29b, miR-29c) have been recognized as tumor suppressors that are downregulated in several types of cancer [8]. In HBV-related HCC, the role of miR-29 remains largely unknown. In a preliminary study, we found that miR-29c was significantly downregulated in HBV-related HCC cell lines and in HBV transgenic mice compared with their corresponding controls. On the basis of these findings, miR-29c was selected for further investigation in the present study. Tumor necrosis factor alpha-induced protein 3 (TNFAIP3), a key regulator in inflammation and immunity, was found to be inversely correlated with miR-29c levels, both in cell lines and in clinical specimens. TNFAIP3 was identified as a target gene that was regulated by miR-29c. We also demonstrated that the overexpression of miR-29c in HepG2.2.15 cells effectively suppressed TNFAIP3 expression and HBV DNA replication, as well as inhibited cell proliferation and induced apoptosis. We conclude that miR-29c may play an important role as a tumor suppressive miRNA in the development and progression of HBV-related HCC by targeting TNFAIP3. Thus, miR-29c and TNFAIP3 represent key diagnostic markers and potential therapeutic targets for the prevention and treatment of HBV infection.

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2. Materials and methods

2.1. Tissues samples

Human liver tissues were obtained with informed consent from patients in Shandong Provincial Hospital, China, which included two normal hepatic tissues as well as 22 pairs of tumors and adjacent non-cancerous tissues (NT). All the patients had been diagnosed with HBV-related HCC. All tissues were flash frozen in liquid nitrogen after surgical resection and stored at −80 °C before use.

2.2. Cell lines

An HCC cell line (HepG2) was cultured in DMEM (Hyclone, China) with 10% FBS (Gibco, USA) and 100 U/ml penicillin and streptomycin. HepG2.2.15 cell line which was transfected with full-length HBV genome was cultured in MEM (Hyclone, China) with 10% FBS and 380 mg/L antibiotic G-418 sulfate (Promega, USA). All cells were cultured at 37 °C in a 5% CO_2 incubator.

2.3. miR-29c expression analysis by qRT-PCR

Reverse transcription and quantitative real-time PCR (qRT-PCR) was performed to measure expression levels for miR-29c in HepG2.2.15 cells, HepG2 cells, HBV-related HCC tissues and NT. Total RNA were isolated using TRIzol Reagent (Invitrogen). All cDNA was synthesized using an NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen). The reactions were performed using a preheated real-time PCR instrument (ABI7000) with the EXPRESS SYBR GreenER™ miRNA qRT-PCR Kit (Invitrogen). All reactions were performed in triplicate. The relative expression levels of miR-29c compared with snRNA U6 were determined using the $2^{-\Delta\Delta C_T}$ method.

2.4. Bioinformatic analysis of miR-29c target genes

Putative miR-29c targets were predicted using several different algorithms, including TargetScan (http://www.targetscan.org/), Pictar (http://pictar.bio.nyu.edu/) and miRanda (http://microrna.sanger.ac.uk/). An interaction between miR-29c and the 3′-UTR of its target gene was predicted by RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/).

2.5. RT-PCR for TNFAIP3

Total RNA was extracted as described above, and 1 μg of total RNA was reversely transcribed to cDNA using the First Strand cDNA Synthesis Kit (Toyobo, Japan). The TNFAIP3 gene was amplified with the following primer pair: forward: 5′-gagagcacaatggctgaaca-3′; reverse: 5′-cacaagcttccggacttctc-3′. The human β-actin gene was used as an endogenous control for RNA normalization and was amplified with the following primer pair: forward: 5′-acactgtgcccatctacgag ggg-3′; reverse: 5′-atgatggagttgaaggtagtttcgtggat-3′. PCR was performed using PCR MasterMix (Tiangen, China). The products were analyzed on a 1.2% agarose gel that was stained with ethidium bromide.

2.6. Construction of miR-29c expression plasmid

The following amplified primer pair was designed based on the cDNA sequence of the *Homo sapiens* miR-29c precursor and included restriction enzyme cut sites as well as protecting bases: 5′catggatcctcgacaccatcagtctgctc3′ (BamHI) and 5′gcgaagcttctgctttt ccccctacatca3′ (HindIII). The PCR products were collected, purified and digested by BamHI and HindIII and then inserted into the pSilencer3.1-H1 neo expression vector that had been digested by the same two restriction enzymes. The recombinant plasmid, named pS-miR-29c, was confirmed by PCR, restriction enzyme digestion and DNA sequencing.

2.7. Transfection

One day before transfection, HepG2.2.15 cells were plated at a density of 2×10^5 cells per well in 6-well plates. Transfection was performed with 2 μg of either pS-miR-29c or pSilencer3.1-control using FuGENE 6 (Roche) according to the manufacturer's instructions. The expression levels of miR-29c were measured in HepG2.2.15 cells after transfection by qPCR, as described above. RT-PCR was used to measure TNFAIP3 expression levels after transfection, as described above. qRT-PCR analysis for TNFAIP3 expression was performed on the ABI7000 system using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen). Each sample was analyzed in triplicate. The data were normalized to β-actin using the $2^{-\Delta\Delta C_T}$ method.

2.8. Luciferase reporter assay

To create a luciferase reporter construct, 3′-UTR segments of TNFAIP3 that contained the putative binding sites for miR-29c were synthesized by PCR using the following primers: forward: 5′-tggtacctatggctaaccggaaacagg-3′(KpnI); reverse: 5′-ggagatctccttg ggctgaatctgacat-3′ (BglII). After appropriate 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-TNFAIP3 construct. HepG2 cells (2×10^5) were seeded on a 24-well plate. Cells were co-transfected with pS-miR-29c and either pGL3-TNFAIP3 or pGL3-control using FuGENE 6 24 h after plating. The samples were also co-transfected with 50 ng of pRL-TK plasmid expressing *Renilla luciferase* to determine the transfection efficiency. Then, cells were harvested 24 h after transfection, and Firefly and *Renilla luciferase* activity levels were measured using the Dual-Luciferase Reporter Assay (Promega). Each transfection was performed in triplicate.

2.9. Western blot analysis

The cells were lysed with 1% RIPA Lysis Buffer (Beyotime) 72 h after transfection. The supernatants were collected, and protein concentration was determined using the BCA Assay Kit (Pierce, Rockford, IL). The protein samples were separated analyzed by 10% SDS–PAGE and then transferred to a PVDF membrane. The membrane was blocked with 5% milk, followed by an overnight incubation at 4 °C with a primary mouse monoclonal antibody against human TNFAIP3 (Abcam, USA). The membrane was washed three times for 5 min in TBST and then incubated with a goat anti-mouse HRP secondary antibody. The membrane was washed three times for 5 min in TBST, and the bound antibody was detected by chemiluminescence with the ECL Detection Reagent (Pierce, Rockford, IL). The data were normalized to β-actin.

2.10. HBsAg and HBeAg assay

The viral protein levels of hepatitis B surface antigen (HBsAg) and e antigen (HBeAg) in transfected cells were measured 48 h after transfection by ELISA (Autobio, Zhengzhou, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm, and the assays were performed in triplicate. Inhibitory rates were calculated according to the following formula: inhibitory rate (%) = (C control − C tested)/C control × 100%.