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miR-342-3p affects hepatocellular carcinoma cell proliferation via regulating NF-κB pathway

Liang Zhao, Yubao Zhang*

Department of Hepatopancreatobiliary Surgery, The Harbin Medical University Cancer Hospital, Harbin 150086, PR China

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ABSTRACT

Recent research indicates that non-coding microRNAs (miRNAs) help regulate basic cellular processes in many types of cancer cells. We hypothesized that overexpression of miR-342-3p might affect proliferation of hepatocellular carcinoma (HCC) cells. After confirming overexpression of miR-342-3p with qRT-PCR, MTT assay showed that HCC cell proliferation was significantly inhibited by miR-342-3p, and that it significantly decreased BrdU-positive cell proliferation by nearly sixfold. Searching for targets using three algorithms we found that miR-342-3p is related to the NF-κB pathway and luciferase assay found that IKK-γ, TAB2 and TAB3 are miR-342-3p target genes. Results of western blot on extracted nuclear proteins of HepG2 and HCT-116 cells showed that miR-342-3p reduced and miR-342-3p-in increased p65 nuclear levels and qRT-PCR found that NF-κB pathway downstream genes were downregulated by miR-342-3p and upregulated by miR-342-3p-in, confirming that miR-342 targets NF-κB pathway. Overexpression of Ikk-γ, TAB2 and TAB3 partially rescued HCC cells proliferation inhibited by miR-342-3p. Using the GSE54751 database we evaluated expression from 10 HCC samples, which strongly suggested down-regulation of miR-342-3p and we also found inverse expression between miR-342-3p and its targets IKK-γ, TAB2 and TAB3 from 71 HCC samples. Our results show that miR-342-3p has a significant role in HCC cell proliferation and is suitable for investigation of therapeutic targets.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and is estimated to be the third leading cause of cancer death worldwide [1]. As HCC is seldom detected in the early stages, survival of HCC patients is as short as a few months [2]. Approximately 90% of HCC cases start with cirrhosis, which can be caused by a wide range of factors including hepatitis B and C infections, alcohol abuse, non-alcoholic fatty liver disease, autoimmune-mediated hepatitis, primary biliary cirrhosis (PBC) and exposure to carcinogens [3]. Considerable progresses on unraveling molecular mechanisms of HCC have been achieved recently, paving the way to the early detection and treatment of HCC.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression by binding to complementary sequence in the 3′-UTR of target mRNAs. Such binding results in either degradation of the target mRNAs or inhibits their translation into proteins [4,5]. miRNAs have been implicated in biological processes including metabolism, cell proliferation, developmental timing, apoptosis, morphogenesis and response to stress [6–8]. Regulation by miRNAs have been found to correlate with cancers, with roles as both oncogenes and tumor suppressors [9,10] and miRNAs are considered potential diagnostic and prognostic biomarkers [11–14] and perhaps therapeutic targets [15,16].

Cittelly et al., published results suggesting that the miRNA miR-342 regulates tamoxifen response in breast cancer cells [17]. Their clinical data showed a trend toward reduced miR-342 expression and tamoxifen resistance, and their study results suggest miR-342 regulates expression of genes involved in tamoxifen-mediated tumor-cell apoptosis and cell cycle progression. They identified miR-342 as an important mediator of tamoxifen response in breast cancer tumor cell lines and breast cancer patients [17].

In an analysis of differential expression of miRNAs in the brains of bovine spongiform encephalopathy-infected cynomolgus macaques as a model for Creutsfeldt-Jakob disease, Montag and colleagues [18] hypothesized that miRNAs are also regulated in response to human prion disease, based on recent evidence that the lack of miRNA processing promotes neurodegeneration, and that

* Corresponding author. Department of Hepatopancreatobiliary Surgery, The Harbin Medical University Cancer Hospital, 150 Haping Road, Nangang District, Harbin 150086, PR China. Fax: +86 451 86298076.
E-mail address: zhyb880077@sina.com (Y. Zhang).

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deregulation of several miRNAs was reported as associated with Scrapie in mice. They found significant upregulation of hsa-miR-342-3p and hsa-miR-494 in the brains of BSE-infected macaques compared with uninfected controls.

FOXM1 is a well-established oncogenic factor shown to be involved in multiple biological processes including cell proliferation, growth, angiogenesis, migration and invasion and can be regulated by miRNAs [19,20]. Li et al. reported that FOXM1 is directly targeted by miR-342-3p, which is down-regulated along with its host gene, EVL, in human cervical cancer tissue, compared to the adjacent normal tissues [21]. Functional studies suggested that the overexpression of miR-342-3p inhibits cell proliferation, migration and invasion in cervical cancer cell lines. FOXM1 was upregulated and negatively correlated with miR-342-3p in cervical cancer tissues, and the overexpression of FOXM1 rescued the phenotype changes induced by the overexpression of miR-342-3p.

In the present study, we sought to determine whether overexpression of miR-342-3p similarly suppresses proliferation of HCC cells. Using the cell lines HepG2 and HCT-116, as well as clinical samples and publically available algorithms, we found that miR-342-3p regulates the NF-κB pathway and thereby suppresses cell proliferation in HCC.

2. Materials and methods

2.1. Data base search and miR-342-3p potential target prediction

The GSE54751 database was used to determine whether miR-342-3p expression is altered in 10 HCC tissues compared to adjacent normal liver tissues, and three algorithms (miRanda, DIANA and TargetScan) were subsequently used to predict potential miR-342-3p targets.

2.2. Cell lines and cultures

HepG2 and HCT-116 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Earle's minimal essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 pg/ml streptomycin and 4 mM glutamine (Invitrogen). All cells were cultured in a 5% carbon dioxide humidified incubator at 37 °C.

2.3. miRNA and transfection

The miR-342-3p, miR-342-3p-in and control mimics were purchased from RiboBio (Guangzhou, Guangdong, China). Cells were transfected with miR-342-3p, miR-342-3p-in or control mimics using Lipofectamine LTX (Invitrogen) according to manufacturer's instructions. Experiments on cells were performed 2 days after transfection.

2.4. MTT assay

Twenty-five hundred HepG2 and HCT-116 cells per well were seeded in 96-well plates. Cell viability was determined by MTT assay after transfection with miR-342-3p or control mimics. Twenty microliter of MTT (Promega, Madison, WI) were added to each well and incubated for 4 h. Absorbance was measured at 540 nm wavelength to measure cell viability.

2.5. (5-Bromo-2′-deoxyuridine) BrdU assay

Transfected cells were seeded on coverslips in 24-well plates and cultured overnight. BrdU (10 μg/ml) was added to the culture medium and cells further incubated for 1 h. Cells were immediately fixed in 4% paraformaldehyde for 10 min and stained with an anti-BrdU antibody (Biocompare, South San Francisco, CA) per manufacturer's instructions. The coverslips were counterstained with DAPI and imaged acquired with fluorescence microscopy (Olympus, Tokyo, Japan). Results were expressed as the percentage of BrdU + cells in DAPI + cells.

2.6. Plasmid constructions, transfections and luciferase activity assay

IKK-α, IKK-β, IKK-γ, IKK-ε, TAB2 and TAB3 3′UTR were generated by PCR amplification and inserted into the pGL3-basic luciferase reporter plasmid (Promega). We purchased NF-κB luciferase reporter plasmid from QIAGEN, Inc. (Valencia, CA). IKK-γ, TAB2 and TAB3 cDNA was amplified by RT-PCR using RNA from 293 cells as template. PCR primers were based on GenBank accession number NM_001099856, NM_001292034 and NM_152787. The entire open reading frame of IKK-γ, TAB2 or TAB3 was cloned into NheI and NotI sites of the mammalian expression vector pcDNA3.1. HCC cells were seeded into 24-well plates and cotransfected with each reporter plasmid and miRNA encoding plasmid using Lipofectamine LTX. Luciferase and renilla signals were measured 2 days after transfection using a Dual Luciferase Reporter Assay Kit (Promega).

2.7. RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR) analysis

HepG2 and HCT-116 cells were transiently transfected with miR-342 control mimics or miR-342-3p for 2 days. Cell were then lysed with TRIzol reagent (Invitrogen) for dissociation of any RNA-protein complexes. Chloroform was added for phase separation, followed by aqueous precipitation of total RNA using isopropyl alcohol. After centrifugation the RNA pellet was washed in 75% ethanol and suspended in nuclease-free TE buffer. Two micrograms of total RNA were reverse transcribed into cDNA using a High-Capacity RNA-to-cDNA kit (Invitrogen). qRT-PCR was performed with primers for miR-342-3p, IKK-γ, TAB2, TAB3 TNF-α, IL-1b, IL-6, IL-8, cyclin d1 and MYC with SYBR in the 7500 HT real-time PCR System (Applied Biosystems, Carlsbad, California). β-actin and U6 were used as endogenous controls for mRNA and miRNA measurements. Results were expressed using the $2^{\Delta\Delta Ct}$ method. The primers used for qRT-PCR measurement were *IKK-γ* forward 5′-CTT TTGGGGTAGATGCG-3′ and reverse 5′-GGTTAAATACACATCGGTCTG-3’; *TAB2* forward 5′-CTCGAAGGCGCCTGAAAAGA-3′ and reverse 5′- GGGTTTTGGTG-GCACAGGAC-3′; *TAB3* forward 5′-CAGCCCACAGCTTGATATTC-3′ and reverse 5′-CATGACTTTGCCCGAGTTAG-3′; *TNF-α* forward 5′-GCCGCATCGCCGTCTCCTAC-3′ and reverse 5′- CCTCAGCCC-CCTCTGGGGTC-3′; *IL-1b* forward 5′-AATCTGTACCTGTCCTGCGTGTT-3′ and reverse 5′-TGGGTAATTTTTGGGATCTACACTCT-3′; *IL-6* forward 5′-TTCTCCACAAGCGCCTTCGGTC-3′ and reverse 5′-TCTGTGTGGGGCGGCTACATCT-3′; *IL-8* forward 5′-GTGCAGTTTTGC-CAAGGAGT-3′ and reverse 5′-CTCTGCACCCAGTTTTCCTT-3’; *cyclin d1* forward 5′-CCGTCCATGCGGAAGATC-3′ and reverse 5′-GAA-GACCTCCTCCTCGCACT-3′; *MYC* forward 5′- GCCACGTCTCCACA-CATCAG-3′ and reverse 5′- TCTTGGCAGCAGGATAGTCCTT-3′; β-actin forward 5′-ATGGGTCAGAA GGATTCCTATGTG-3′ and reverse 5′-CTTCATGAGGTAGTCAGTCAGGTC-3’.

2.8. Western blot

Proteins in HepG2 and HCT-116 cells 2 days after miR-342-3p transfection were extracted with RIPA lysis buffer (Applygen, Beijing, China) mixed with a proteinase inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). The total protein concentrations

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