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MicroRNA-101 suppresses SOX9-dependent tumorigenicity and promotes favorable prognosis of human hepatocellular carcinoma

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ABSTRACT

We previously showed that high expression levels of SOX9 correlate with hepatocellular carcinoma (HCC) progression. However, the exact role that SOX9 plays in HCC remains unclear. In this study, we firstly confirmed that miRNA-101 directly targets SOX9 in HCC. Ectopic expression of miR-101 significantly inhibited HCC cell proliferation and tumorigenicity by targeting SOX9. Moreover, the down-regulation of miR-101 in clinical HCC tissues correlates with tumor aggressiveness and poor prognosis. Therefore, miR-101 may suppress HCC tumor progression by down-regulating SOX9. MiR-101 may be a potential prognostic marker and therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancies and the third leading cause of cancer-related deaths worldwide [1]. The distribution of HCC is unbalanced throughout the world, with the highest incidence in Asia and Sub-Saharan Africa, especially in China. As a notoriously aggressive solid tumor, HCC is characterized by fast infiltrating growth, early metastasis, high-grade malignancy, and poor prognosis. This cancer often develops as a consequence of underlying liver diseases such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, and is almost associated with cirrhosis [2]. At present, surgical hepatic resection and liver transplantation are the only curative treatment modality to confer survival benefit in HCC patients. However, the overall 5-year survival rate for HCC patients is still only 5% [3]. Therefore, it is necessary to elucidate the molecular mechanisms underlying HCC progression and identify novel therapeutic targets in order to improve the clinical outcome of patients with this cancer.

MicroRNAs (miRNAs), a class of genes encoding small RNA molecules (19–22 nucleotides), play important roles in regulating protein expression by inhibiting translation or inducing mRNA degradation by binding to the 3′-untranslational region (3′-UTR) of target mRNAs [4]. Beyond the involvement in diverse physiological and pathological processes, the increasing evidences have well demonstrated that deregulation or dysfunction of miRNAs can contribute to carcinogenesis and cancer development [5]. The roles of miRNAs in different cancers may be dependent on cancer type [6]. Some miRNAs may act as oncogenes overexpressing in cancers and contributing to the transformed phenotypes. These oncogenic miRNAs function by suppressing tumor suppressor genes. Other miRNAs may act as tumor suppressors down-regulating in cancers and allowing the expression of oncogenes. As far as HCC is concerned, accumulating evidence indicates that deregulation of miRNAs can contribute to HCC development by influencing cell growth, apoptosis, migration, or invasion. Aberrant miRNA expression has also been demonstrated to associate with clinical features of HCC, such as stage, differentiation, prognosis, and response to adjuvant therapy [7,8]. Therefore, it is necessary to identify more miRNAs as prognosis predictor or therapeutic target for this cancer.

The transcription factor SOX9 (sex determining region Y [SRY] related high-mobility group box 9) is a member of the SRY (sex

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determining region Y) box gene superfamily [9]. It is essential in sex determination, chondrogenesis, respiratory epithelium development, melanocyte differentiation, and the differentiation of Paneth cells in the gut [10]. Recent studies have detected the expression of SOX9 in multiple tissues during embryogenesis, including cartilage, neural crest, notochord, kidney, pancreas, and endocardial cushions of the heart [11]. Besides these findings, SOX9 has also been demonstrated to play a role in a variety of malignancies. For example, Wang et al. [12] detected the expression of SOX9 in prostate cancer cells contributes to tumor growth and invasion; Aleman et al. [13] found that SOX9 hypermethylation in primary bladder tumours was present more than half of the cases and was significantly associated with tumour grade and overall survival; Malki et al. [14] shown that the embryonic male prostaglandin D synthase/SOX9 pathway was expressed at both the RNA and protein levels in different types of human ovarian tumors, pointing to SOX9 as a possible diagnostic marker for ovarian carcinomas. Especially, the previous study of our group detected the up-regulation of SOX9 at mRNA and protein levels in clinical HCC tissues compared with their adjacent non-neoplastic tissues [15]. Our data also indicated that high SOX9 expression is associated with advanced tumor progression and poor clinical outcome of HCC patients [15]. On the basis of these findings, the aim of the present study was to elucidate the mechanism of SOX9 plays in HCC.

2. Materials and methods

2.1. Cell culture

Human HCC cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA, USA) and was cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibico, USA), 2 mM L-glutamine and antibiotics. Normal human liver cell line HL-7702 was obtained from the American Type Culture Collection (Manassas, VA, USA) and was maintained in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibico, USA). Two cell lines were both maintained at 37 °C in a humidified chamber supplemented with 5% CO_2.

2.2. Patients and tissue samples

The study was approved by the Research Ethics Committee of 302nd Hospital of PLA, Beijing, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 130 self-pairs of HCC specimens and adjacent non-neoplastic liver tissues were snap-frozen in liquid nitrogen and stored at −80 °C following surgery for qRT-PCR assay. All the tissues were obtained from 130 patients with primary HCC who underwent a curative liver resection at the 302nd Hospital of PLA, Beijing, China. These patients were diagnosed as HCC between 2001 and 2006. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. HCC diagnosis was based on World Health Organization (WHO) criteria. Tumor differentiation was defined according to the Edmondson grading system. Liver function was assessed using the Child-Pugh scoring system. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. The clinicopathological features of 130 patients are summarized in Table 1.

The median follow-up period was 8.6 years. Postoperative surveillance included routine clinical and laboratory examinations every third month, computed tomography scans of the abdomen, and radiographs of the chest every third month. After 5 years, the examination interval was extended to 12 months.

Table 1
Clinicopathological features and the expression of miRNA-101 in 130 hepatocellular carcinoma patients.

Clinicopathological features	Case	MiRNA-101 expression frequency (*n*, %)		*P*
		High	Low	
Age (years)				
⩽50	72	33 (45.83)	39 (54.17)	NS
>50	58	22 (37.93)	36 (62.07)	
Gender				
Male	96	40 (41.67)	56 (58.33)	NS
Female	34	15 (44.12)	19 (55.88)	
Tumor stage				
T1	23	23 (100.00)	0 (0)	0.02
T2	40	20 (50.00)	20 (50.00)	
T3	52	12 (23.08)	40 (76.92)	
T4	15	0 (0)	15 (100.00)	
Tumor grade				
G1	31	31 (100.00)	0 (0)	0.008
G2	76	24 (31.58)	52 (68.42)	
G3	23	0 (0)	23 (100.00)	
Growth pattern				
Trabecular	101	44 (43.56)	57 (56.44)	NS
Non-trabecular	29	11 (37.93)	18 (62.07)	
Cirrhosis				
Yes	86	36 (41.87)	50 (58.13)	NS
No	44	19 (43.18)	25 (56.82)	
Underlying liver disease				
Alcoholic	25	11 (44.00)	14 (56.00)	NS
Hepatitis B	49	20 (40.82)	29 (59.18)	
Hepatitis C	35	15 (42.86)	20 (57.14)	
Unknown	21	9 (42.86)	12 (57.14)	

2.3. Target prediction

An online program Target-Scan (release human 6.2) was used for predicting miRNAs that might target SOX9.

2.4. EGFP-miR-101 expression vector and cellular transfection

We obtained the commercial Pre-miR-101 expression vector (human pMIR101-1PA-1) with the following sequence: 5′-TGC CCT GGC TCA GTT ATC ACA GTG CTG ATG CTG TCT ATT CTA AAG GTA CAG TAC TGT GAT AAC TGA AGG ATG GCA-3′, Pre-miR-145 expression vector as a positive control with the following sequence: 5′- CAC CUU GUC CUC ACG GUC CAG UUU UCC CAG GAA UCC CUU AGA UGC UAA GAU GGG GAU UCC UGG AAA UAC UGU UCU UGA GGU CAU GGU U-3′, and the same negative-vector as a negative control from System Bioscience (Mountain View, CA). A fluorescent marker (GFP) is also present to monitor cells that are positive for transfection. HepG2 cells were transfected through Fugene transfecting agents (Roche) with hsa-miR-101 vector, hsa-miR-145 vector (positive control), and negative control (NC) following the manufacturer's instructions, respectively.

2.5. qRT-PCR

The qRT-PCR analysis for miRNA was performed according to the similar protocol of the previous studies. Briefly, total RNA, including miRNA, was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After that, the total RNA was reversely transcribed using the corresponding RT Primer and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). U6 small RNA was used as an internal control for normalization and quantification of the target miRNA expression. The specificity of amplification was confirmed by melting curve analysis and also by running PCR products on agarose gels