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MicroRNA-645 represses hepatocellular carcinoma progression by inhibiting SOX30-mediated p53 transcriptional activation

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ABSTRACT

Amount of evidence demonstrate that aberrant microRNAs (miRNAs) are involved in tumorigenesis and progression in hepatocellular carcinoma (HCC). Among them, miR-645 is recently recognized as cancer-related miRNA and its significance in HCC remains largely unknown. In this study, we reported for the first that miR-645 expression was markedly elevated in HCC tissues and cell lines, and its up-regulation was associated with malignant clinical features, including tumor size and venous infiltration and poor prognosis. Our data revealed that miR-645 promoted cell proliferation, colony formation and inhibited apoptosis by gain- and loss-of function experiments *in vitro*. *In vivo* assays showed that miR-645 overexpression enhanced tumor growth. Moreover, miR-645 directly bound to the SOX30 3′-UTR and post-transcriptionally repressed SOX30 expression in HCC cells. Furthermore, miR-645 inversely correlated with SOX30 expression in HCC tissues. Restoration of SOX30 expression at least partially abolished the biological effects of miR-645 on HCC cells. SOX30 regulated HCC progression through aberrant activation of p53 by directly binding to its promoter. Taken together, this research supports the first evidence that miR-645 exerts an oncogenic role in HCC progression and may be a therapeutic target for HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and a leading cause of cancer-related mortalities worldwide [1,2]. Liver resection remains the most common one of curative therapies for HCC patients. In spite of great advancement in the therapy regimen for HCC, the long-term prognosis remains poor due to the high rate of recurrence and metastasis [3,4]. In addition, the underlying mechanisms remain largely unknown. Therefore, it is urgent to uncover the molecular pathways underlying HCC progression and identify the novel biomarkers predicting the post-surgical prognosis of HCC patients.

MicroRNAs (miRNAs), as a class of small non-coding RNAs (ncRNAs), are approximately 22 nucleotides in length, which act as post-transcriptional regulators by binding to the 3′-untranslated regions (UTRs) of their target mRNAs to induce mRNA degradation or translational inhibition [5–7]. Accumulating evidence confirmed that miRNAs play an important role in cell growth, metabolism, invasion, growth, differentiation and metastasis [8]. Recently, miR-645 has been recognized as a cancer-related miRNA in diverse cancers [9,10]. miR-645 promotes metastasis of osteosarcoma *via* targeting tumor suppressor NM23 nucleoside diphosphate kinase 2 [11]. Down-regulation of miR-645 suppresses breast cancer cell metastasis *via* targeting DCDC2 [12]. miR-645 is up-regulated in human adenocarcinoma of gastric esophageal junction inhibits apoptosis by targeting tumor suppressor IFIT2 [13]. miR-645 promotes cell metastasis and proliferation of renal clear cell carcinoma by targeting GK5 [14]. However, the functional role of miR-645 and its downstream target are still unclear in HCC progression.

In present study, we disclosed that miR-645 was up-regulated in HCC tissues and cell lines. Its overexpression was associated with poor prognostic features and reduced survival of HCC patients. MiR-645 promoted the growth of HCC cells *in vitro* and *in vivo*. Furthermore, we verified that SRY-related HMG-box 30 (SOX30) was a direct target of miR-645. Moreover, SOX30 directly binds to p53 promoter region and activates p53 transcription. Overall, our results confirm that miR-645 is a novel regulator in HCC progression and represents a potential target for HCC diagnosis and treatment.

2. Materials and methods

2.1. Clinical samples and cell culture

HCC and adjacent corresponding normal tissues were obtained from the First Affiliated Hospital of Xi'an Jiaotong University. None of the

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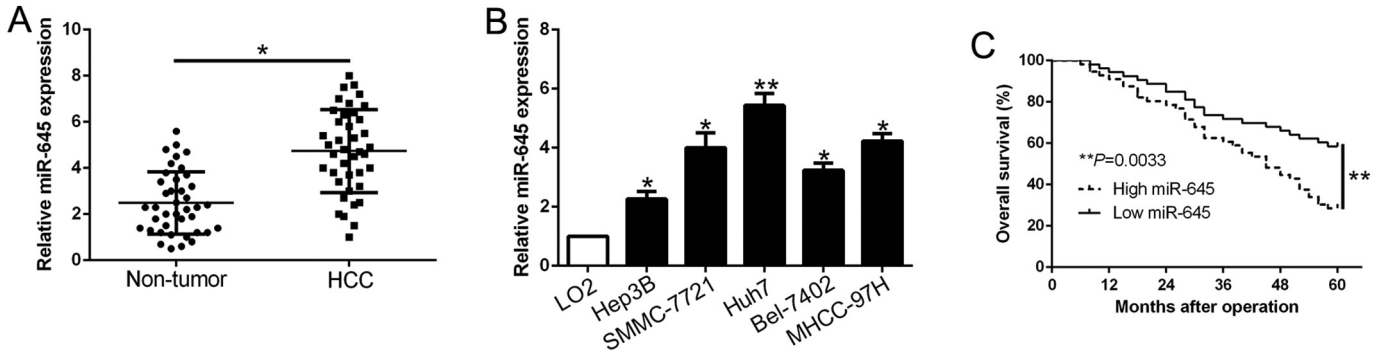

Fig. 1. Expression of miR-645 in HCC. (A) The expression level of miR-645 was determined in tumor tissues and adjacent normal tissues of HCC patients. (B) miR-645 expression was up-regulated in HCC cell lines compared to normal hepatic cell line LO2. (C) Kaplan–Meier analyses of the overall survival in HCC patients with high or low levels of miR-645. *P < 0.05, **P < 0.01.

patients received radiofrequency ablation, radiotherapy or chemotherapy before surgery. After surgical resection, the tissues were frozen in liquid nitrogen immediately and stored at −80 °C until further analyses. Written consent was collected from all patients and the study was approved by the Ethics Committee of the Xi'an Jiaotong University.

The normal immortalized human hepatocyte LO2 and a panel of HCC cells (Hep3B, Bel-7402, Huh7, MHCC-97H and SMMC-7721) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) at 37 °C in humidified atmosphere with 5% CO_2.

Table 1
Clinical correlation of miR-645 expression in HCC (n = 109).

Clinical parameters	Cases (n)	Expression level		P value (*P < 0.05)
		miR-645^{high} (n = 56)	miR-645^{low} (n = 53)	
Age (years)				
<50 years	42	21	21	0.820
≥50 years	67	35	32	
Gender				
Male	84	43	41	0.943
Female	25	13	12	
Tumor size (cm)				**0.015***
<5 cm	86	39	47	
≥5 cm	23	17	6	
Tumor number				0.912
Solitary	95	49	46	
Multiple	14	7	7	
Edmondson				
I + II	81	41	40	0.787
III + IV	28	15	13	
TNM stage				0.119
I + II	88	42	46	
III + IV	21	14	7	
Capsular infiltration				0.504
Present	92	46	46	
Absent	17	10	7	
Venous infiltration				**0.041***
Present	16	12	4	
Absent	93	44	49	
AFP				0.879
<400 ng/ml	24	12	12	
≥400 ng/ml	85	44	41	
HBsAg				0.793
Positive	100	51	49	
Negative	9	5	4	

Bold indicates significance value P < 0.05.

2.2. Cell transfection and reagent

Vectors mediated miR-645 (HmiR0351) and miRNA inhibitors (HmiR-AN0755), and their corresponding control vectors (CmiR0001-MR04 and CmiR-AN0001-AM02) were purchased from GeneCopoeia Inc. (Guangzhou, China). For SOX30 overexpression, a full-length human SOX30 cDNA was amplified by PCR and subcloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA). Small interfering RNA (siRNA) used for SOX30 silencing and non-targeting (NT) siRNA were purchased from GenePharma (Shanghai, China). Lipofectamine 2000 Reagent (Invitrogen) was used for cell transfection. HCC cells were harvested for further analysis 48 h after transfection.

2.3. Cell proliferation, colony formation and flow cytometry of apoptosis assay

Edu, colony formation and apoptosis were carried as described previously [15,16].

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA of HCC tissues and cells was isolated using Trizol reagent (Invitrogen) according to its instructions and was reverse transcribed into cDNA using a TakaRa PrimeScript™ RT kit (Takara, Dalian, China). The expression of miR-645 was quantified by TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA). qRT-PCR of SOX30 mRNA was carried out using SYBR Green Premix PCR Master Mix (Roche, Mannheim, Germany) in a StepOnePlus real-time PCR system (Applied Biosystems). The relative expression of miR-645 and SOX30 mRNA was normalized to U6 and GAPDH using $2^{-\Delta\Delta Ct}$ method.

2.5. Western blot analysis

Western blotting was performed according to the protocol described previously [17–19]. The protein concentration was measured using spectrophotometer (BIO-RAD, Hercules, CA), then equal protein was separated by SDS-PAGE and transferred to PVDF membranes. Subsequently, the PVDF membranes were probed with primary antibody over night at 4 °C, then probed with HRP-conjugated secondary antibodies (Cell Signaling Technology). The western blot was detected