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The tumor suppressive miR-26a regulation of FBXO11 inhibits proliferation, migration and invasion of hepatocellular carcinoma cells

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ABSTRACT

Accumulating researches identify microRNA-26a (miR-26a) as a tumor suppressor in hepatocellular carcinoma (HCC). F-box protein 11 (FBXO11), a predicted target gene of miR-26a, is an E3 ubiquitin ligase and a type II methyltransferase, and functions as a key regulator of tumor initiation and progression. This study was aimed to investigate the regulatory role of miR-26a in FBXO11 expression and explored the clinical significance as well as functional role of FBXO11 in HCC. The expression levels of miR-26a were prominently downregulated in HCC tissues compared to matched tumor-adjacent tissues. MiR-26a inversely regulated FBXO11 abundance in HCC cells. Hereby, miR-26a could directly target 3′UTR of FBXO11 mRNA to suppress its expression. Gene Expression Omnibus (GEO) database (GSE54236 and GSE45436) and our data demonstrated that the expression of FBXO11 was up-regulated in HCC tissues. The level of FBXO11 mRNA was inversely correlated with miR-26a expression in HCC specimens. High FBXO11 expression was positively correlated with large tumor size, venous infiltration and advanced tumor stage of HCC patients. Clinical prognostic analysis illustrated that high FBXO11 expression predicted a poor survival of HCC patients. *In vitro*, FBXO11 knockdown inhibited cell proliferation, colony formation, migration and invasion of HCC cells. Additionally, miR-26a overexpression showed a consistent effect with FBXO11 knockdown on these malignant behaviors of HCC cells. Notably, FBXO11 restoration reversed the inhibitory effect of miR-26a on HCC cell proliferation, colony formation, migration and invasion. In summary, these results indicated that miR-26a regulation of FBXO11 exhibited an oncogenic role in HCC. Inhibition of FBXO11 might serve as a therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC), the fifth most common cancer, exhibits extremely high morbidity and mortality rates worldwide [1]. In 2012, more than approximately 700,000 died of HCC, which accounts for 9.1% in all cancers [2]. Tumor recurrence and metastasis is the main cause for the poor survival rate of advanced stage HCC patients [3]. Therefore, identification of novel biomarkers for early stage detection and novel potential therapeutic target of HCC is desirable and urgently needed.

MicroRNAs (miRNAs), as a family of small noncoding 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 [4]. Recent studies have shown the association between various miRNAs and HCC progression. For example, miR-187-3p, miR-542-3p, miR-1296 *etc.* function as tumor suppressors by targeting different genes [5–7], while miR-324-3p acts as an oncogene in HCC [8] and miR-367 promotes cell proliferation and metastasis during the progression of human HCC [9]. MiR-26a has been generally reported as a tumor suppressor of HCC in previous studies. MiR-26a/b induces apoptosis and increases chemosensitivity of HCC cells by suppressing autophagy [10]. MiR-26a exhibits inhibitory effect on cell invasion, migration and growth by targeting ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (ST3GAL6) in HCC [11]. Furthermore, Yang X et al. report that miR-26a suppresses angiogenesis, growth and metastasis of HCC by inhibiting different targets [12,13]. However, novel target that mediates tumor suppressive role of miR-26a deserves to be explored.

Abbreviations: HCC, hepatocellular carcinoma; miRNAs, microRNAs; UTRs, untranslated regions; ST3GAL6, ST3 beta-galactoside alpha-2,3-sialyltransferase 6; FBXO11, F-box protein 11; PRMT9, protein arginine methyltransferase 9; AJCC, American Joint Committee on Cancer; qRT-PCR, quantitative real-time PCR; CCK-8, Cell Counting Kit-8; HIF-1α, hypoxia-inducible factor-1α; GEO, Gene Expression Omnibus, TCGA, The Cancer Genome Atlas

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F-box protein 11 (FBXO11), also known as protein arginine methyltransferase 9 (PRMT9), functions as both an E3 ubiquitin ligase and a type II methyltransferase [14,15]. For instance, FBXO11 is reported to methylate the splicing factor SAP145 [16,17]. Meanwhile, FBXO11 targets SNAIL for ubiquitination and protesomal degradation in cancer progression [18,19]. MiRNAs have been reported to be upstream regulators of FBXO11. MiR-21 promotes tumorigenesis by directly targeting FBXO11 [20]. MiR-621 increases chemosensitivity of breast cancer cells to paclitaxel and carboplatin by inhibiting FBXO11 [21]. However, the expression and regulatory mechanism of FBXO11 and its association with HCC patients' survival are still largely unknown.

In the current study, we were aimed to investigate the expression of miR-26a and FBXO11 in both HCC and tumor-adjacent tissues, and disclosed the effects of FBXO11 on proliferation, migration and invasion of HCC cells. MiR-26a underexpression and FBXO11 overexpression were observed in HCC tissues. FBXO11 was recognized as a novel target of miR-26a and it might mediate the inhibitory effect of miR-26a on HCC cell proliferation, migration and invasion.

2. Materials and methods

2.1. Patient tissue specimens

Eighty paired HCC and adjacent normal tissues were obtained from the Department of Hepatobiliary and Pancreatic Surgery, Affiliated Hospital of Beihua University (Jilin, China). All samples were confirmed by professional pathologists. HCC and adjacent normal tissues were conserved in liquid nitrogen for subsequent qPCR and immunoblotting analysis. All patients did not receive therapies including radiotherapy, chemotherapy and radiofrequency ablation prior to surgery. Tumor staging was determined by the seventh edition of staging system of American Joint Committee on Cancer (AJCC) issued in 2010. The study was approved by Ethic Committee of the Affiliated Hospital of Beihua University (Jilin, China) and written informed consent was signed by all patients.

2.2. Cell culture and transfection

The human HCC cell lines (HepG2 and MHCC97H, HCCLM3) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (HyClone, Logan, UT, USA). All cell lines were incubated in a humidified atmosphere with 5% CO2 at 37 ˚C.

The miR-26a mimics, miR-26a inhibitors and their corresponding negative control were purchased from GeneCopoeia (Guangzhou, China). FBXO11 shRNA, non-targeting (NT) shRNA and FBXO11 expression plasmid (pcDNA3.1-FBXO11) were constructed and purchased from Ribobio (Guangzhou, China). Cells transfection was performed by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The shRNA targeting sequence was listed as follow: 5′-GAGTTTACATCTTTGGTGA-3′.

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

The total RNA from HCC cells and tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized by TaqMan miRNA reverse transcription (Applied Biosystems, Foster City, CA, USA) and a PrimeScript Reverse Transcriptase kit (Takara, Dalian, China). The relative expression of miR-26a and FBXO11 mRNA were quantified using miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems) and the SYBR Premix Ex Taq™ Kit (Takara, Shiga, Japan) in the Applied Biosystems 7500 Sequence Detection system. The relative expression of miR-26a and FBXO11 mRNA were normalized by U6 small nuclear RNA and GAPDH, respectively. The primers of miR-26a (RT primer: GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GCC TA; Forward primer: CCG CCG TTC AAG TAA TCC AG; Reverse primer: AGT GCA GGG TCC GAG GTA TT), U6 (Forward primer: CGC TTC GGC AGC ACA TAT AC; Reverse primer: CAG GGG CCA TGC TAA TCT T), FBXO11 (Forward primer: GAT GGA CGA GGC CTT ATT GA; Reverse primer: TGT TAT GCC GAA CAA TTG GA) and GAPDH (Forward primer: TGC ACC ACC AAC TGC TTA GC; Reverse primer: GGC ATG GAC TGT GGT CAT GAG) were designed and synthesized by Sangon Biotech (Shanghai, China).

2.4. Western blotting

Western blotting was performed according to the protocol described previously [22]. 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 antibody against FBXO11 (ab181801, Abcam, Cambridge, MA, USA) and GAPDH (sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA, USA), then probed with HRP-conjugated secondary antibodies (#7074 and #7076, Cell Signaling Technology, Beverly, MA, USA). The western blot was detected with enhanced chemiluminescence regents (Thermo Scientific, Waltham, MA, USA).

2.5. Luciferase reporter assay

The sequence of FBXO11 3′UTR containing the putative miR-26a-5p binding region was amplified from human genomic DNA. Then the sequence was cloned into pGL3 luciferase reporter vector (Promega, Madison, WI, USA) (wild type FBXO11 3′UTR vector). The potential miR-26a-5p binding sites were mutated by the Quick-change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) (mutant FBXO11 3′UTR vector). The wild type FBXO11 3′UTR vector or mutant FBXO11 3′UTR vector and miR-26a mimics or miR-26a inhibitors were co-transfected into HCCLM3 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) under luminometer (Berthold Detection System, Pforzheim, Germany), and luciferase activity was normalized to Renilla activity.

2.6. Cell counting Kit-8 (CCK-8) proliferation assay

Cell growth was examined using Cell Counting Kit-8 assay (CCK8, Dojindo Molecular Technologies, Inc., Kunamoto, Japan). Briefly, MHCC97H and HCCLM3 cells were placed in a 96-well plate in triplicate with 2×10^3 cells/well. CCK-8 reagent was added to each well to measure cell proliferation at 24, 48 and 72h. The spectrophotometer (Thermo Scientific, Rockford, IL, USA) was used to detect the cell proliferation at the absorbance of 490nm.

2.7. Colony formation analysis

HCCLM3 and MHCC97H cells were seeded into 12-well plate in triplicate with 1×10^3 cells/well and then cultured for 10 days in a 5% CO2 incubator at 37 ˚C. Furthermore, cells were washed three times by PBS, then fixed by 4% paraformaldehyde and stained with 0.1% crystal violet for 20 min. The number of cell colonies in the each filed was counted.

2.8. Cell migration and invasion assay

HCCLM3 and MHCC97H cells were plated in a 6-well plate 24 h post-transfection and then cultured for 24 h until 90% confluence. A scratch was created on the cells using a sterile 200 µl micropipette tip. The cells were subsequently rinsed twice with serum-free medium to