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Let-7b contributes to hepatocellular cancer progression through Wnt/β-catenin signaling

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ABSTRACT

Elevated evidences show that microRNAs (miRNAs) play vital roles in tumor progression regulation. However, the functional role of let-7b in hepatocellular carcinoma (HCC) is still largely unknown. In this study, we try to investigate the biological activity of let-7b in human HCC cells and try to find the potential regulatory signaling pathway. Our results indicate that let- 7b was remarkably down-regulated in human HCC tissues by qRT-PCR. In addition, let-7b overexpression decreased the expression of β-catenin and c-Myc, while upregulated E-cadherin expression in HCC cells which was verified by quantitative real-time PCR (qRT-PCR) and western blotting. Furthermore, Wnt/β-catenin was involved in let-7b biological activity which was revealed by luciferase assay. Moreover, Wnt/β-catenin signaling inhibitor blocks HCC cell proliferation which is as the same pattern as let-7b overexpression inhibits in HCC cells proliferation. In conclusion, down-regulated let-7b promotes HCC cell proliferation through Wnt/β-catenin signaling in HCC cells. These results suggested that appropriate manipulation of let-7b might be a new treatment of human HCC in the future.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and has an increasing incidence worldwide (Parkin et al., 2001; Parkin et al., 2005; Llovet and Bruix, 2008). China is one of the areas of the world with high incidence for HCC and contributes to 55% of all HCC cases (Lai et al., 2003). The curative treatments for early-stage HCC are liver transplantation and liver resection. Multistep processes, accumulation of genetic and epigenetic alterations, are included in HCC carcinogenesis. However, the molecular pathogenesis is not well understood (Chu et al., 2014; Hoshida et al., 2009). Thus, it is imperative to identify new possible targets for preventing the initiation and progression of HCC.

MicroRNAs (miRNAs) are non-coding small RNAs (~21–23nt) that regulate various biological processes by binding to the 3′-untranslated region (3′-UTR) of their target mRNAs, which cause either mRNA degradation or translational repression (Engels and Hutvagner, 2006; Iwakawa and Tomari, 2013). MiRNAs play regulatory roles in multiple cellular processes, including cell proliferation, apoptosis and differentiation (Gibb et al., 2011; Bjorner et al., 2014). Accumulating evidence has shown that both losses and gains of miRNA function are associated with oncogenic transformation. The role of miRNAs in carcinogenesis/cancer has also been discussed. For example, tumor-associated miRNAs have been identified in the serum of patients with diffuse large B-cell lymphoma (Calin et al., 2002; Lawrie et al., 2008). Currently, miRNAs have made them attractive therapeutic targets for cancer molecular therapy. Although much is known about the profiles of miRNAs in many tumors and tissues, the function of miRNAs in HCC has not yet been fully elucidated.

Wnt/β-catenin signaling has been shown to regulate the progression of cancer because it promotes cell proliferation and migration. β-catenin protein is the downstream molecule of Wnt/β-catenin signaling which is accumulated in the nuclear location after WNT1 binds to specific Frizzled (FZD) surface receptors. E-cadherin and c-Myc have been reported to be involved in the β-catenin regulatory network (Thievessen et al., 2003; Zhang et al., 2012; Qualtrough et al., 2015; Su et al., 2015; Xu et al., 2016). The activated Wnt/β-catenin signaling subsequently activates distinct various cellular signaling pathways critical for cancer

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development (Ito et al., 2000; Masaki et al., 2003). Several clinical studies have reported that abnormal activation of Wnt/β-catenin pathway is contributed to hepatocarcinogenesis (Endo et al., 2000; Inagawa et al., 2002). Thus, it is attractive to further explore the effects of Wnt/β-catenin signaling on HCC.

In conclusion, in this study, we verified that let-7b is down-regulated in HCC. Further investigation showed that Wnt/β-catenin signaling activity was suppressed by let-7b in vitro. Additionally, Wnt/β-catenin signaling inhibition blocks the effects on HCC cell proliferation caused by let-7b knockdown. Our results indicate that let-7b may be a new therapeutic target in HCC and add another layer on miRNA biology.

2. Material and methods

2.1. Cell culture

Human HCC cell line HepG2 was purchased from American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin in a humidified incubator at 37 °C containing 5% CO2.

2.2. RNA isolation and quantitative real-time PCR (qRT-PCR)

Extraction of total RNA from cells was in accordance to the instruction of mirVana™ miRNA Isolation Kit (Thermo Scientific). For isolation of intact RNA from tissues, tissues were minced on ice, frozen in liquid nitrogen immediately and stored at −80 °C until analyzed.

Intact RNA from tissues after homogenization was prepared using mirVana™ miRNA Isolation Kit (Thermo Scientific). Total RNAs were eluted with 100 μl of elution buffer. TaqMan microRNA assay was used to determine levels of mature miRNAs in tissues or cells according to the instruction of TaqMan microRNA reverse transcription kit (Thermofisher).

qRT-PCR is the most sensitive technique for mRNA and miRNA detection. TaqMan qRT-PCR assays were used for the detection of gene/mature miRNA expression in triplicate on an ABI 7500 system (Applied Biosystems, Foster City, CA). All the gene/miRNA-specific probes were bought from Applied Biosystems.

2.3. Overexpression and knockdown of let-7b

To evaluate the functional effect of let-7b, we overexpressed let-7b in HepG2 cell in vitro. We purchased lentiviral let-7b (LV-let-7b) and empty lentiviral vector (LV-VC) from Applied Biological Materials Inc (Richmond, BC). After then, the let-7b and VC overexpression lentiviruses were stored at −80 °C until use. Lentiviral transfection efficiency (>90%) was confirmed by fluorescent microscopy.

To block the signaling regulation caused by let-7b, let-7b inhibitor (GeneCopoeia, Guangzhou, China) assay was performed as previous description (Thomson et al., 2013).

2.4. Wnt/β-catenin activity reporter assay

To monitor Wnt/β-catenin signaling pathway activity in cells, Wnt/β-catenin signaling pathway luciferase reporter assay was performed as previous description (Zhang et al., 2015). In brief, HepG2 cells were seeded overnight in a 96-well plate (2 × 104 cells/well). On the next day, cells were co-transfected with 50 ng let-7b or either a control miRNA (Con-miR) with 100 ng Wnt/β-catenin pathway-luciferase-reporter construct (SABiosciences, Frederick, MD). MiRNA plasmids were constructed as previous description (Zhang et al., 2015). 24 h after transfection, Wnt3a conditioned medium (Wnt3a-CM) were replaced the old medium. After 18 h stimulation, the cells were harvested. Wnt/β-catenin signaling activity was determined by Dual Luciferase Reporter Assay System (Promega, San Luis Obispo, CA). WNT inhibitory factor 1 (WIF1) (Sigma-Aldrich, St Louis, MO) was used to inhibit Wnt/β-catenin signaling, Data was represented as the ratio of firefly luciferase activity to Renilla luciferase activity.

2.5. Western blotting

Western blot is often used to separate and identify proteins. After cells were lysed in lysis buffer (Thermo Scientific, Waltham, MA), protein concentrations were determined using DC Protein Assay (Bio Rad Laboratories, Hercules, CA). Western blot analysis was according to a previous description with 15ug of proteins per well (Zhang et al., 2015). For specific primary antibodies (Ab): anti- β-catenin (1:500; Sigma-Aldrich, St Louis, MO), anti–c-Myc (1:500 dilution; Cell Signaling Technologies, Beverly, MA), anti-E-cadherin (1:1000, Cell signaling) and anti–β-actin (1:500; Sigma-Aldrich) following the addition of the correlated HRP-conjugated secondary Abs (Abcam). In order to avoid incomplete stripping, all of the results are from separate membranes.

2.6. Cell viability assay

Cell viability of HepG2 cell was determined by Cell Counting Kit-8 (CCK8) method according to the manufacture's protocol (Promega). Brief, cells were seeded in 96-well plates at densities of 5000 cells/ well and transfected with 50 nM let-7b or con-miR for 48 h. At the end of transfection, 100 μl fresh medium was added to replace the medium used for transfection. Cells were treated with CCK8 dye at days 0–7 of culture. The absorbance at 450 nm was then measured in a microplate reader. Cell viability was calculated by comparing with blank control (BC) cells. BC cells

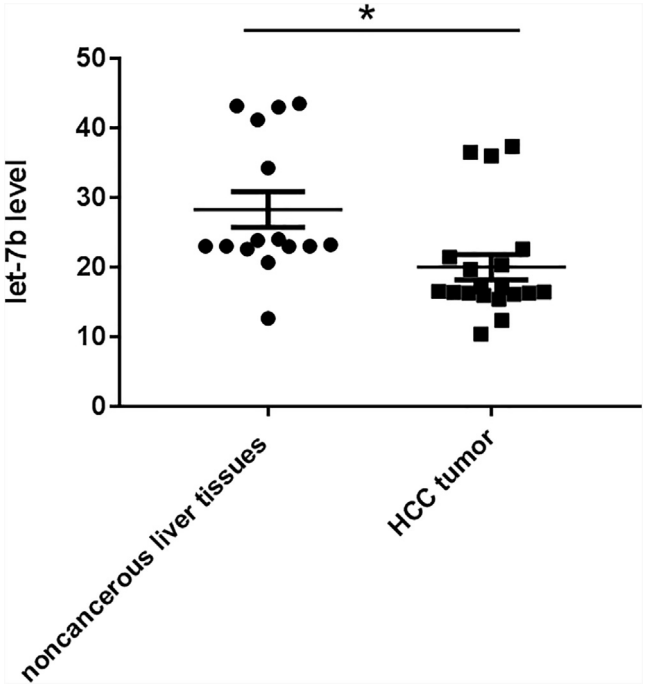

Fig. 1. Let-7b expression level in HCC tumor and noncancerous liver tissues. Let-7b expression level was determined by qRT-PCR. U6 was used as an endogenous control for the quantification. Results are mean ± SD. $^{*}p < 0.05$ vs HCC tumor.