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MiR-214 inhibits cell growth in hepatocellular carcinoma through suppression of β-catenin

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ABSTRACT

Mounting evidence has shown that microRNAs (miRNAs) are implicated in carcinogenesis and can function as oncogenes or tumor suppressor genes in human cancers. Recent profile studies of miRNA expression have documented a deregulation of miRNA (miR-214) in hepatocellular carcinoma (HCC). However, its potential functions and underlying mechanisms in hepatocarcinogenesis remain largely unknown. Here, we confirmed that miR-214 is significantly downregulated in HCC cells and specimens. Ectopic overexpression of miR-214 inhibited proliferation of HCC cells in vitro and tumorigenicity in vivo. Further studies revealed that miR-214 could directly target the 3′-untranslated region (3′-UTR) of β-catenin mRNA and suppress its protein expression. Similar to the restoring miR-214 expression, β-catenin downregulation inhibited cell growth, whereas restoring the β-catenin expression abolished the function of miR-214. Moreover, miR-214-mediated reduction of β-catenin resulted in suppression of several downstream genes including c-Myc, cyclinD1, TCF-1, and LEF-1. These findings indicate that miR-214 serves as tumor suppressor and plays substantial roles in inhibiting the tumorigenesis of HCC through suppression of β-catenin. Given these, miR-214 may serve as a useful prognostic or therapeutic target for treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. Despite great advances in HCC treatment, HCC remains the third leading cause of cancer-related death, and the 5 years survival rate of patients is less than 5% [2]. Therefore, it is necessary to elucidate the underlying molecular mechanisms of HCC and develop novel strategies for the diagnosis, treatment and prognosis of HCC.

MicroRNAs (miRNAs) are an abundant class of non-coding short RNAs that suppress target gene expression at the post-transcriptional level by inducing mRNA degradation or suppression of translation [3,4]. Recently, an increasing number of reports have indicated that the aberrant expression of miRNAs correlates with various human cancers, including human HCC, and that miRNAs can function as tumor suppressors and oncogenes [5]. This provides new avenues for HCC diagnostic and therapeutic application. So far, multiple human miRNAs have been shown to be dysregulated in HCC, such as miR-7, miR-124, miR-122, miR-26a, and miR-155 [6–10], which contribute to the development and progression of HCC.

Notably, previous studies have documented that miR-214 is downregulated in HCC tissues compared with adjacent normal tissues by miRNA microarray analysis [11,12]. It has been shown that miR-214 is aberrantly expressed in cervical cancers and inhibits the growth of HeLa cells [13]. Additionally, miR-214 has been reported to suppress cell proliferation and invasion in breast cancer by targeting EZH2, a bonafide oncogene [14]. More recently, Shih and collegues reported that miR-214 downregulation contributes to tumor angiogenesis by inducing secretion of the hepatoma-derived growth factor in human hepatoma [15]. These studies suggest a potential tumor suppressive function of miR-214. However, until now, the role of miR-214 in hepatocarcinogenesis and the underlying molecular mechanisms by which miR-214 exerts its functions are largely unknown.

In this current study, we investigated the potential involvement of miR-214 in HCC. We demonstrated significant downregulation of miR-214 in HCC cell lines and tissues. Furthermore, overexpression of miR-214 potently inhibited HCC cell growth both in vitro and in vivo. More importantly, we provide evidence, for the first

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time, that β-catenin is a direct and functional target of miR-214. Our data suggest that miR-214 may be a potential therapeutic target for treating HCC.

2. Materials and methods

2.1. HCC tissue samples and cell cultures

Eighteen HCC and adjacent normal tissues were obtained from patients in First Affiliated Hospital of Fujian Medical University. The tissues were immediately snap-frozen in liquid nitrogen after surgical removal and stored at −80 °C. All protocols were approved by the Ethics Committee of Fujian Medical University. HCC cell lines Hep3B, SK-Hep1, SMMC-7721, Huh7, HepG2 and the normal hepatic cell line LO2 were provided by Institute of Biochemistry and Cell Biology of Chinese Academy of Science (China) and originated from ATCC. All cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, Maryland) supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C under 5% CO_2 humidified atmosphere.

2.2. Lentivirus production and infection

The pre-miR-214 sequence and open-reading frame of β-catenin were amplified and cloned into the pCDH-CMV-MCS-EF1-coGFP lentiviral vector (System Biosciences, California, USA). Virus packaging and infection were performed according to standard protocols as recommended by the manufacturer. The packaged lentiviruses were named Lv-miR214, Lv-β-catenin, and Lv-shβ-catenin. The empty lentiviral vector was used as a control. The primers are the following: pre-miR-214, 5′-ATAGAATTCTTT CTCCCTTTCCCCTTACTCTCC-3′ (forward) and 5′-CCAGGATCCTTT CATAGGCACCACTCACTTTAC-3′ (reverse), and β-catenin, 5′-CGC TCTAGAATGGCTACTCAAGCTGATTTGATGG-3′ (forward) and 5′-CCA GGATCCTTACAGGTCAGTATCAAACCAGGCC-3′ (reverse).

2.3. RNA extraction and quantitative real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. TaqMan MiRNA Assays (Applied Biosystems) were performed to determine the expression level of miR-214. Equal amounts of RNA was reverse transcribed with a miRNA-specific primer, followed by real-time PCR with TaqMan probes. U6 snRNA was used as an internal control. For β-catenin mRNA analysis, the first-strand cDNA was synthesized using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) and measured on an ABI 7900 HT system. The PCR primers for β-catenin were 5′-AAAATGG CAGTGCGTTTAG-3′ (forward) and 5′-TTTGAAGGCAGTCTGTCGTA-3′ (reverse). The expression level of β-actin was used as an endogenous control. All samples were normalized to endogenous control and determined using the $2^{-\Delta\Delta Ct}$ analysis method.

2.4. Plasmid construction and luciferase reporter assay

The 3′UTR of β-catenin gene, containing one putative miR-214 binding site, was cloned downstream of a cytomegalovirus (CMV) promoter-driven firefly luciferase cassette in a pCDNA3.0 vector. The primer sequences are for β-catenin 3′UTR were: 5′-CGCTCTA-GAGGTAGGGTAAATCAGTAAGAGGTGT-3′ (forward) and 5′-TAGGC CGGCCTACTACGAAGTTCTCTTTTACGCCAAT-3′ (reverse). The mutant constructs were generated by either deletion or mutation. For the luciferase assay, HepG2 and Huh7 cells were cultured in 24-well plates and co-transfected with 400 ng DNA of β-catenin 3′UTR wt plasmid or β-catenin 3′UTR mt plasmid in the presence of either miR-214 or miR-control. After 48 h, luciferase activity was measured using the dual luciferase reporter assay system (Promega, Madison, WI). The experiments were performed independently in triplicate.

2.5. Cell proliferation assay

Cells were seeded in 96-well plate at 4000 cells per well and maintained in culture medium containing 10% fetal bovine serum for 6 days. The viable proliferation cells were determined by MTT assay. The absorbance at 570 nm (OD570) was detected using μQuant Universal Microplate Spectrophotometer (Bio-tek Instruments, Winooski, VT, USA).

2.6. Cell cycle analysis

Cells were collected and fixed in 70% ethanol at 4 °C overnight. After the ethanol was removed, the cells were incubated with 1 mg/ml RNase A in PBS for 30 min, and then the cells were incubated an additional 30 min in the dark in 0.5 ml of 50 mg/ml propidium iodide (PI). The distribution of cells throughout the cell cycle was analyzed by Cell Lab Quanta SC flow cytometry (Beckman Coulter, Fullerton, CA) and the data were analyzed by FlowJo v7.6 Software.

2.7. Western blotting

Cells were lysed with RIPA lysis buffer and proteins were harvested. Total cell protein extracts were separated by 10% SDS polyacrylamide gel electrophoresis, and then transferred onto a nitrocellulose membrane (Millipore, USA). After blocking with 5% non-fat milk, the blots were incubated with primary antibodies against β-catenin, CyclinD1, c-Myc, TCF-1, LEF-1 and β-actin (Abcom, Cambridge, UK) at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, signals were developed with Super Signal West Pico chemoluminescent substrate (Pierce, Rockford, IL), visualized by the GeneGnome HR Image Capture System (Syngene, Frederick, MD). The intensity of protein fragments was quantified using Image-Pro Plus software.

2.8. In vivo xenograft tumor growth in nude mice

Four-week-old, male Balb/c athymic nude mice were housed and manipulated according to the protocols approved by Fujian Medical Experimental Animal Care Commission. Approximately 5×10^6 HepG2-miR214 or control cells in log phase were injected subcutaneously into the dorsal flank of the mice. The mice were housed for 25 days post inoculation. Then all the mice were euthanized and the tumor tissues were removed by surgical excision. Tumor volume was measured every 5 days during the experimental period.

2.9. Statistical analysis

Data are expressed as the mean ± SE of at least three independent experiments. The differences between groups were analyzed using two-sided Student's *t*-tests. Data were considered to be statistically significant when $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. miR-214 is frequently downregulated in human HCC cell lines and tissues

We first examined the expression levels of miR-214 in five HCC cell lines and 18 pairs of HCC and adjacent non-neoplastic liver tissues. The results of qRT-PCR showed that all five HCC cell lines (i.e., HepG2, SMMC-7721, Huh7, SK-Hep1, Hep3B) had lower levels of miR-214 expression than that in the normal hepatic cell line LO2 (Fig. 1A). Consistent with these data, the average expression level of miR-214 was significantly lower in HCC specimens than in paired adjacent non-neoplastic liver tissues (Fig. 1B). Taken together, these results suggest that the expression of miR-214 is significantly decreased in human HCC specimens and cell lines.

3.2. miR-214 overexpression inhibits HCC cell growth in vitro and in vivo

In an attempt to determine the impact of miR-214 on HCC cell growth, we established HepG2 and HuH-7 transfectants stably expressing miR-214 using lentivirus infection. Successful increased expression of miR-214 in these established cell lines was confirmed by qRT-PCR (Fig. 2A). As shown in Fig. 2B, the results of MTT assay revealed that overexpression of miR-214 significantly inhibited cell growth in HepG2 cells by 35% and in HuH-7 cells by 25%, whereas the miR-control had no effect on cell growth (Fig. 2B). Furthermore, we examined cell-cycle distribution in these cells. Compared with miR-control, miR-214-overexpressing HepG2 and HuH-7 cells displayed an increased percentage of cells in G0/G1 phase and fewer cells in S phase (Fig. 2C). These results indicate that miR-214 may inhibit cell proliferation by inducing cell cycle arrest in G0/G1 phase. To further confirm the above findings in vivo, HepG2 cells stably expressing miR-214 or control cells were injected subcutaneously into the right scapula of nude mice and the animals were closely monitored for tumor growth. As shown in Fig. 2D and E, miR-214-overexpressed tumors showed a slower growth and a significantly decreased volume compared to the vector control tumors. The average tumor weight was also significantly reduced in miR-214-overexpressed tumors compared to the controls (Fig. 2F). Taken together, these results suggest that miR-214 functions as a growth suppressor in human HCC.

3.3. miR-214 directly targets β-catenin 3′UTR in HCC cells

To understand the underlying mechanism of growth inhibition induced by miR-214, we searched for miR-214 targets using TargetScan. Our analysis revealed that β-catenin was a potential target of miR-214. The 3′-UTR of β-catenin mRNA contains a complementary site for the seed region of miR-214 (Fig. 3A). To determine whether β-catenin is a direct target of miR-214, a human β-catenin 3′-UTR fragment containing wild-type (wt 3′UTR) or mutant (mt 3′UTR) miR-214 binding sequence was cloned downstream of the reporter gene (Fig. 3A). HCC HepG2 and HuH-7 cells were then co-transfected with wt or mt 3′UTR vector. As shown in Fig. 3B, the relative luciferase activity of the reporter containing wt 3′-UTR was significantly suppressed following miR-214 transfection. However, site-directed mutagenesis of the miR-214 binding site within β-catenin's 3′-UTR completely abolished this suppression, suggesting that miR-214 directly binds to this site. Furthermore, we examined the changes of β-catenin expression in HCC HepG2 and HuH-7 cells after miR-214 overexpression. The results showed that overexpression of miR-214 significantly decreased the levels of β-catenin protein, whereas its mRNA level was constant (Fig. 3C and D). To further elucidate whether the growth-suppressive effect of miR-214 was mediated by repression of β-catenin in HCC cells, we performed gain-of-function and loss-of-function studies. As shown in Fig. 3E and F, knockdown of β-catenin by shβ-catenin led to significant cell growth inhibition similar to that induced by miR-214; transfection with β-catenin open reading frame plasmid without 3'-UTR (cannot be targeted by miR-214) significantly rescued miR-214-induced cell growth inhibition. Taken together, these results strongly suggested that β-catenin is a direct target of miR-214 in HCC cells.

3.4. miR-214 regulates β-catenin downstream signaling molecules

Given that miR-214 suppressed β-catenin expression, the next question was whether the expression of β-catenin downstream pathway was also impacted by miR-214. To this end, the levels of β-catenin downstream genes implicated in tumor growth, such as CyclinD1, c-Myc, and TCF-1, were examined by western blotting. As shown in Fig. 4A, the expression of these molecules was all reduced in HepG2 and Huh7 cells stably overexpressing miR-214 in vitro. Accordingly, the expression of CyclinD1, c-Myc, and LEF-1 was also reduced in the xenograft tumor tissues with miR-214 overexpression (Fig. 4B). Taken together, these results suggest that miR-214 induces the expression of β-catenin downstream genes in HCC cells.

4. Discussion

Recently, miRNAs have emerged as critical regulators of carcinogenesis and tumor progression in HCC [16,17]. In the present

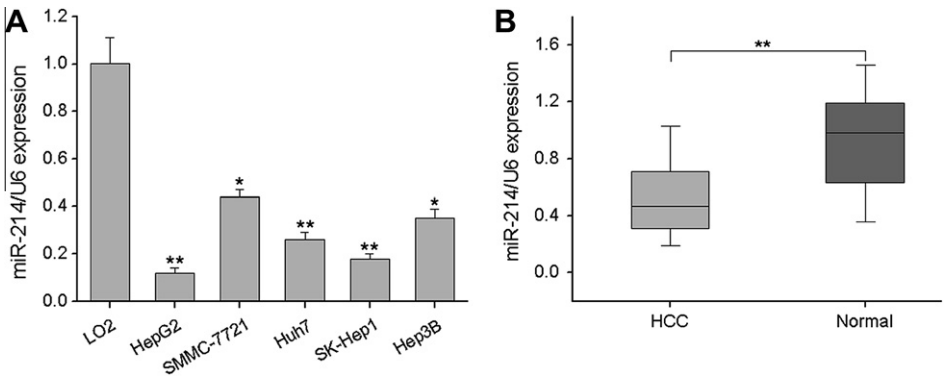

Fig. 1. Expression of miR-214 in HCC cell lines and tissues. (A) The expression level of miR-214 in normal hepatic cells and five HCC cell lines was examined by qRT–PCR. Data are presented as mean ± SE ($^{*}p < 0.05$, $^{**}p < 0.01$, independent t test) and, (B) The relative expression of miR-214 in 18 paired HCC and adjacent non-tumor tissues. Alteration of expression is expressed as box plot presentations, with the y-axis indicating miR-214 expression.

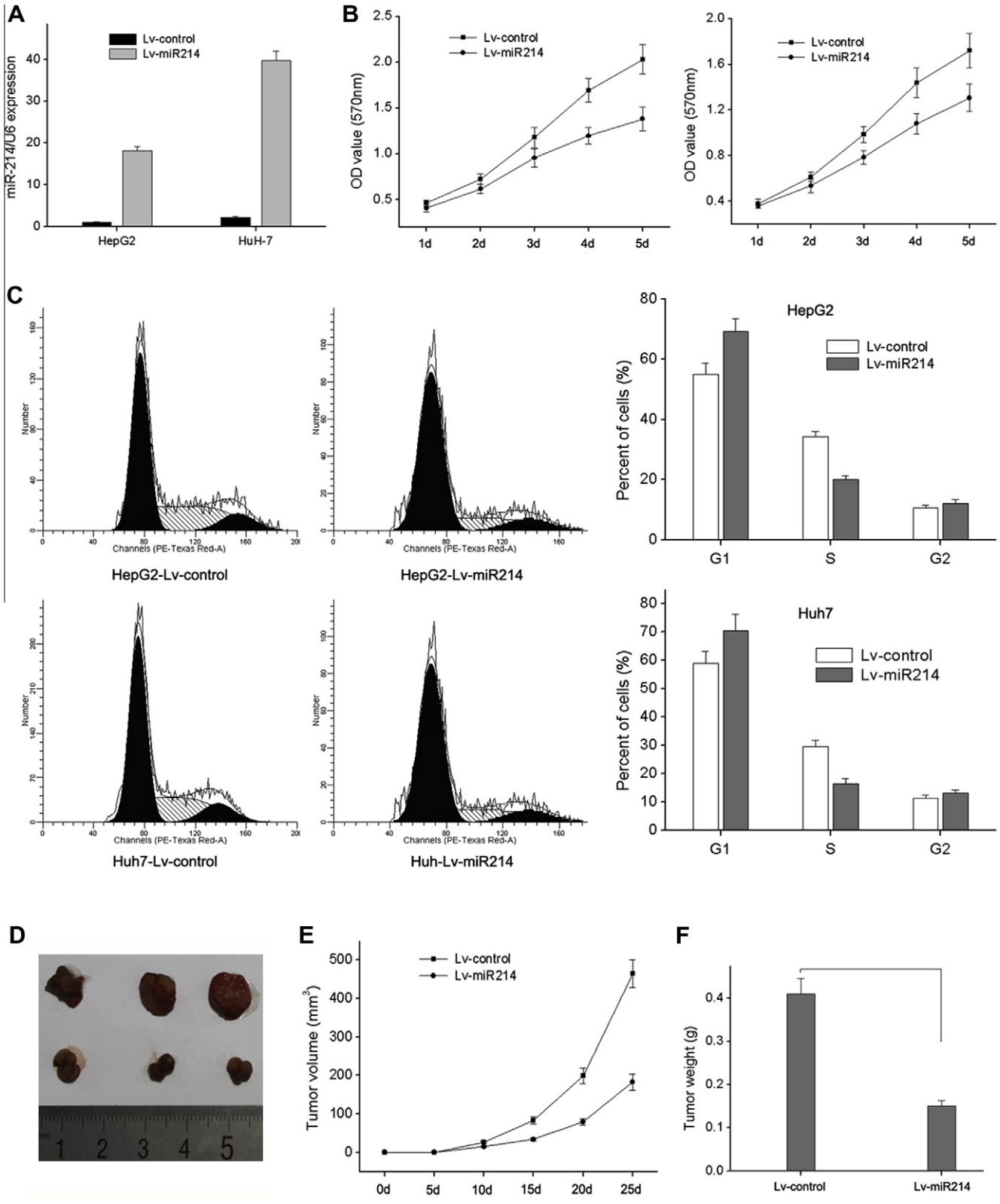

Fig. 2. Enforced expression of miR-214 inhibits HCC cell growth in vitro and in vivo. (A) Expression of miR-214 in cells infected with miR-214 lentivirus (indicated as Lv-miR214) and control lentivirus (indicated as Lv-control) was measured by qRT-PCR. (B) Cell proliferation assay (MTT). (C) Cell cycle analysis. (D) Photography of xenograft tumors recovered from three representative nude mice. miR-214-overexpressed or scramble control HepG2 cells (5×10^6) were inoculated subcutaneously into eight nude mice, and the mice were closed monitored for tumor growth. 25 days post inoculation, the mice were sacrificed and the tumors were recovered and, (E) Growth curve of tumor volumes. (F) The weight of xenograft tumors. Each data point represents the mean ± SE of three mice.

study, we demonstrate that miR-214 acts as a tumor-suppressor in HCC. We observed that downregulation of miR-214 is a frequent event in HCC tissues and cell lines. Ectopic overexpression of miR-214 could inhibit cell proliferation, induce a G0/G1 arrest in HCC cells, and suppress tumor growth in a murine model of HCC xenograft. These results are similar to the findings in cervical and

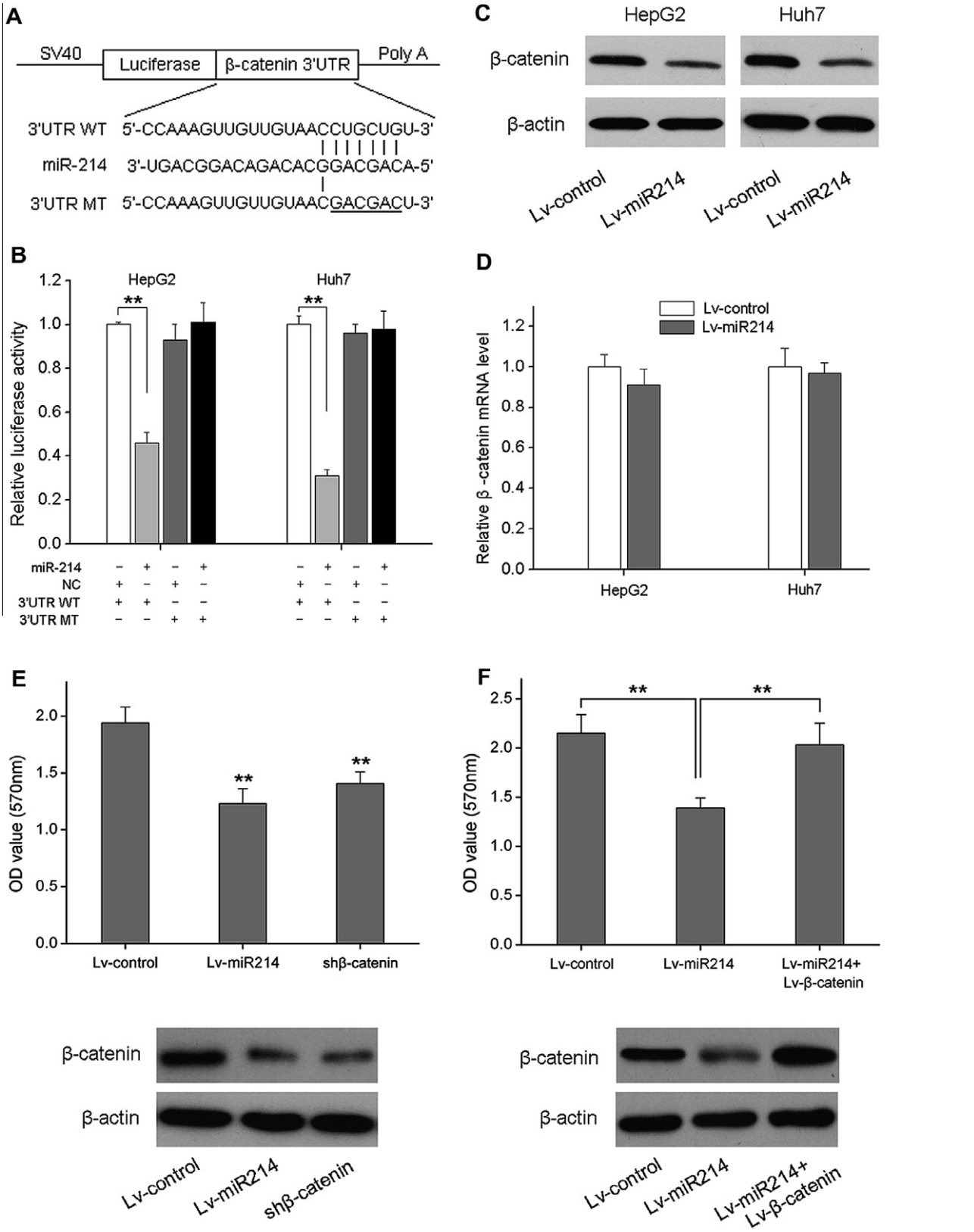

Fig. 3. β-catenin is a direct target of miR-214 in HCC cells. (A) Diagram of β-catenin 3′-UTR-containing reporter construct. Mutation was generated on its 3′-UTR sequence in the complementary site for the seed region of miR-214, as indicated. (B) Analysis of luciferase activity. The wild-type or mutant reporter plasmids were co-transfected into HepG2 and Huh7 cells which were infected with Lv-miR214 or Lv-control. Luciferase activity was assayed 48 h after transfection. Columns showed the mean of at least three independent experiments done in duplicate ($^{**}P < 0.05$, independent t test). (C) The expression of β-catenin protein was analyzed by using western blotting. (D) The expression of β-catenin mRNA in HepG2 and Huh7 cells was analyzed by qRT-PCR. (E) HepG2 cells were infected with Lv-shβ-catenin or Lv-miR214. Then cells were analyzed for proliferation by MTT assay. (F) HepG2 cells were infected with Lv-miR214 for 72 h, followed by infection with Lv-β-catenin. Cell growth rate was then performed. Data represent mean ± SE from three individual experiments. Representative western blots for β-catenin in cells transfected with shβ-catenin or expression lentivirus are also shown.

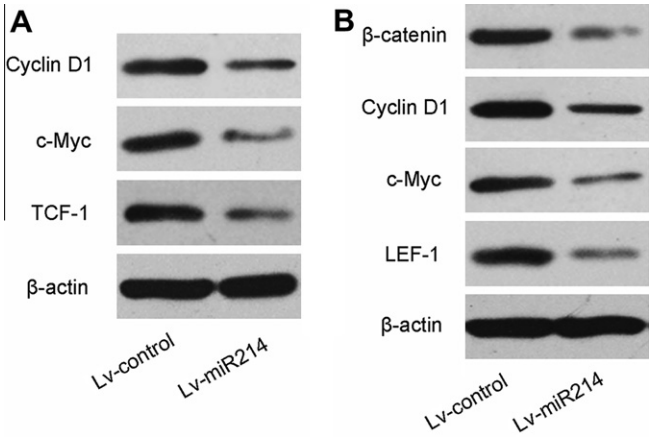

Fig. 4. The effect of miR-214 on Wnt/β-catenin downstream molecules. (A) Analysis of the expression of Cyclin D1, c-Myc and TCF-1 in HepG2 cells infection with Lv-control or Lv-miR214 by western blotting. (B) Analysis of the expression of β-catenin, Cyclin D1, c-Myc and LEF-1 in xenograft tumor tissues derived from miR-214-overexpressed or vector control HepG2 cells. β-actin was used as the loading control.

breast cancer [13,14,18], in which miR-214 is downregulated, and overexpression of miR-214 suppresses cell growth. However, miR-214 is usually upregulated in other human cancers including ovarian, stomach cancers and malignant melanomas [19–21]. These controversial results suggested that the role of miR-214 is possibly tumor specific and highly dependent on its targets in different cancer cells.

Our data also demonstrate that β-catenin is a direct functional target of miR-214 in HCC cells. There are several lines of evidence to support this. First, complementary sequence of miR-214 is identified in the 3′UTR of β-catenin mRNA. Overexpression of miR-214 decreased β-catenin 3′UTR luciferase report activity and this effect was abolished by mutation of the miR-214 seed binding site. Second, miR-214 overexpression reduced β-catenin protein and this effect was attenuated by miR-214 knockdown. These data strongly suggest that β-catenin was a direct target of miR-214 in HCC cells. To further reveal the functions of β-catenin in HCC, we found that knockdown of β-catenin significantly inhibits cell growth, and β-catenin overexpression could rescue the growth suppressive effect of miR-214. These results suggest that the growth inhibitory effect of miR-214 is partly mediated by suppression of β-catenin expression.

Human β-catenin, a homolog of the Drosophila Armadillo protein, is a multifunctional protein that plays essential roles in development and tissue maintenance [22]. It is well known that β-catenin signaling pathway plays a essential role in the pathogenesis of various cancers, including HCC [23,24]. To further explore the molecular mechanisms of growth inhibition induced by miR-214, we examined the expression levels of β-catenin downstream genes, such as CyclinD1, c-Myc, and TCF-1 [25–28]. The results indicated that the protein levels of these molecules were all significantly suppressed, suggesting that miR-214 may be an important regulator of this signaling pathway. Importantly, we found that these downregulated expression of miR-214 effectors could be restored by overexpression of β-catenin. Consistent with these observations, the levels of CyclinD1, c-Myc, and LEF-1 were also reduced in the xenograft tumor tissues with miR-214 overexpression. All these results document that miR-214 suppresses β-catenin expression and downregulates β-catenin downstream genes, which, in turn, inhibites the growth and tumorigenicity of HCC cells.

In summary, we have reported the tumor suppressive role of miR-214 in human HCC. The expression levels of miR-214 is significantly downregulated in HCC cells and tissues. Overexpression of miR-214 can inhibit HCC cell growth in vitro and tumorigenesis in vivo. Furthermore, β-catenin is a direct and functional target of miR-214. miR-214-mediated HCC cell growth inhibition is achieved through suppression of β-catenin and its downstream genes. Given these, restoration of miR-214 expression may be a potential therapeutic strategy for the treatment of HCC in the future.

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