

MicroRNA-320a downregulation mediates human liver cancer cell proliferation through the Wnt/ β -catenin signaling pathway

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Abstract. MicroRNAs (miRs) have emerged as key epigenetic regulators involved in cancer progression. miR-320a has been demonstrated to be a novel tumor suppressive microRNA in several types of cancers. In the present study, the role of miR-320a in human hepatocellular carcinoma (HCC) was investigated. The expression levels of miR-320a and messenger RNA were determined by reverse transcription-quantitative polymerase chain reaction, while cell cycle and cell apoptosis were analyzed by flow cytometry. The cell proliferative ability was determined by Cell Counting Kit-8 assay and colony formation assay. The downstream target of miR-320a was confirmed by luciferase reporter assay, while the protein levels were measured by western blotting. The results revealed that miR-320a was inversely associated with HCC proliferation in HCC cell lines. Functional studies demonstrated that miR-320a significantly decreased the capability of cell proliferation and induced G₀/G₁ growth arrest *in vitro*. In addition, β -catenin was identified as one of the direct targets of miR-320a, downregulating the expression level of β -catenin, c-myc, cyclin D1 and dickkopf-1. In conclusion, miR-320a may act as a tumor-suppressive microRNA through targeting β -catenin in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with >0.7 million newly diagnosed cases annually. The disease ranks as the second most frequent cause of cancer-associated mortality. HCC is a lethal disease, which causes ~0.75 million mortalities per year, and half of these occur in China (1,2). Although therapies such as surgery or chemotherapy are employed, patients with HCC have a high rate of recurrence due to invasion and metastasis (3).

Therefore, there is an urgent requirement to find novel targets for the development of novel effective therapies for HCC.

MicroRNAs (miRs) are 22- to 25-nucleotide single-stranded non-coding RNAs that bind to the 3'-untranslated regions (3'-UTRs) of target mRNA, which results in mRNA degradation (4). MicroRNAs are involved in numerous physiological processes, including cell differentiation, proliferation, metabolism and apoptosis (5-7). MicroRNAs have also been found to play an important role in tumor development via the regulation of oncogene and tumor suppressor expression, or by directly acting as oncogenes or tumor suppressors (8-10). In HCC, microRNAs such as miR-200a, miR-125b and miR-214 have been found to be highly expressed in aggressive tumors, while certain other microRNAs, such as miR-155, miR-183 and miR-550a, are downregulated in the tumors (11-13). A recent study also demonstrated that microRNA signatures could be a subgroup of potential prognostic biomarkers in HCC patients (14). Notably, miR-320a has been found to be a metastatic suppressor in several types of cancer. Several studies have shown that miR-320a functions as a tumor suppressor via the targeting of subunit α -1 in HCC, neuropilin 1 and Rac1 in colorectal cancer (CRC) cells, and aquaporin 1 and 4 in cerebral ischemia (15-18). A previous study also showed that miR-320a inhibits the Wnt/ β -catenin signaling pathway by targeting the 3'-UTR of β -catenin messenger RNA (mRNA) (19). As miR-320a regulates the expression of multiple targets, it may play a key role in the regulatory network for disease development.

Thus far, the effects of miR-320a in HCC have not been completely elucidated. Hence, it is of great significance to investigate the functions and mechanisms of miR-320a in HCC. In the present study, the potential involvement of miR-320a in liver cancer was investigated. The expression level of miR-320a in HCC tissues and liver cancer cells was examined, and its effects on cell growth, cell cycle distribution and colony formation were tested *in vitro*. Furthermore, the underlying mechanism of miR-320a in liver cancer cells was investigated, which may provide novel insights into the understanding of liver cancer.

Materials and methods

Cell culture. All the cell lines used in the present study were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HEK 293T cells, HL-7702 (normal hepatocellular cells), and the HCC cell lines SMMC-7721, BEL-7402 and HepG2, were cultured in Dulbecco's modified

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Eagle's medium (DMEM). All media were supplemented with fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) to a final concentration of 10% and with antibiotics; the cells were incubated at 37°C with 5% CO₂.

Tissue samples. This study was approved by the Ethics Review Committees of the Second Hospital of Longyan, Longyan, Fujian, China), and written informed consent was obtained from all patients. A total of 35 patients (23 males and 12 females) with HCC underwent routine surgery (complete resection) at the Second Hospital of Longyan between January 2013 and September 2014. The mean age of the patients was 52.7 years (range, 41-67 years). HCC samples and matched normal liver tissues (located ~2 cm apart) taken from these 35 patients were snap-frozen in liquid nitrogen for further quantitative polymerase chain reaction (qPCR) analysis.

RNA extraction and reverse transcription-qPCR (RT-qPCR). Total RNA and microRNA fractions were isolated from tissues samples and the HL-7702, SMMC-7721, BEL-7402 and HepG2 cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA (1 µg) was reverse transcribed into cDNA using PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), which contained PrimerScript reverse transcriptase, RNase inhibitor, deoxynucleotide mixture and reaction buffer. MicroRNA extraction was performed using the microRNA Extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). RT-qPCR was performed with SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.), under the following cycling conditions: 40 cycles of 58°C for 20 sec and 75°C for 10 sec. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for the mRNA, and RNU6B was used as the microRNA reference. Primers for miR-320a (forward, 5'-GGGCTAAAAGCTGGGTTGA-3' and reverse, 5'-CAG TCGTGTCTGGAGT-3') and β -catenin (forward, 5'-AAA ATGGCAGTGCCTTTAG-3' and reverse, 5'-TTTGAAGGC AGTCTGTCTGTA-3') were obtained from RiboBio (Guangzhou, China). The expression of mRNA and microRNA was normalized to its relative control by the comparative $\Delta\Delta C_q$ method (20). The experiment was performed in triplicates.

Lentiviral transfection for stable expression clones. Plasmid LV3-pGLV-H1-GFP+Puro, with hsa-miR-320a mimics or hsa-miR-320a inhibitor, or their respective control oligonucleotides, namely miR-320a and miR-negative control (NC), and anti-miR-320a and anti-miR-NC, were purchased from GenePharma (Shanghai, China). Lentivirus transfection was performed as per the manufacturer's instructions to establish miR-320a-expressing stable clones (HepG2/miR-320a) and anti-miR-320a-expressing stable clones (HepG2/anti-miR320a) in HepG2 cells. The relative control clones (HepG2/miR-NC and HepG2/anti-miR-NC) were constructed by similar methods.

Luciferase reporter assay. Prediction of miR-320a binding sites was performed using TargetScan software (<http://www.targetscan.org>). Bioinformatics analysis revealed a potential miR-320a binding site for miR-320a in the 3'-UTR region of β -catenin. HEK 293T cells at 60% confluence were transfected with 200 ng DNA from the β -catenin-wild-type

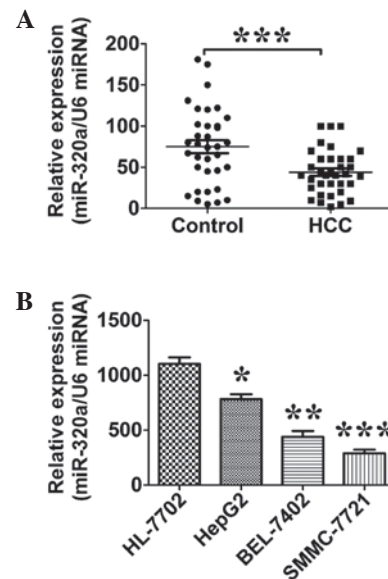


Figure 1. Expression levels of miR-320a in liver cancer cell lines and HCC tissues. (A) Expression levels of mature miR-320a in 35 paired HCC and adjacent non-tumor liver tissues (*** $P<0.001$, unpaired t-test tissues study). (B) Expression levels of miR-320a were examined by reverse transcription-quantitative polymerase chain reaction in HL-7702 cells and three liver cancer cell lines. Data represents the mean \pm standard error of the mean of 3 replicates. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. HL-7702 cells (one-way analysis of variance followed by Bonferroni's multiple comparison tests). miR, microRNA; HCC, hepatocellular carcinoma.

(WT)-UTR plasmid (firefly luciferase reporter vector containing the β -catenin 3'-UTR) or β -catenin-WT-UTR DNA (firefly luciferase reporter vector containing the β -catenin 3'-UTR mutant) and 2 ng pRL-TK vector (Promega Corporation, Madison, WI, USA) in combination with miR-320a mimics (final concentration of 100 nM; GenePharma) or miR-NC (100 nM). Transfection was performed with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific Inc.). The firefly and Renilla luciferase activities were measured by consecutively using Dual Glo Luciferase assays (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase for each transfected well.

Cell cycle analysis. For cell cycle analysis, the cells were harvested after transfection for 24 h. The cells were then fixed with 75% ethanol at 4°C overnight, washed with cold phosphate-buffered saline and treated with RNase I, followed by a 30-min staining with propidium iodide in the dark. Cell cycle distributors were analyzed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit (Abcam, Cambridge, MA, USA) and analyzed by flow cytometry as per the manufacturer's instructions.

Cell proliferation and colony formation assay. Cell proliferation was examined using a water-soluble tetrazolium salt assay via Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Kumamoto, Japan). Briefly, the cells were seeded in 96-well culture plates and incubated at 37°C with 5% CO₂ for 4 days. Once the assay began, 10 µl CCK-8 solution was added to the medium and then incubated at 37°C for 2 h. Cell numbers

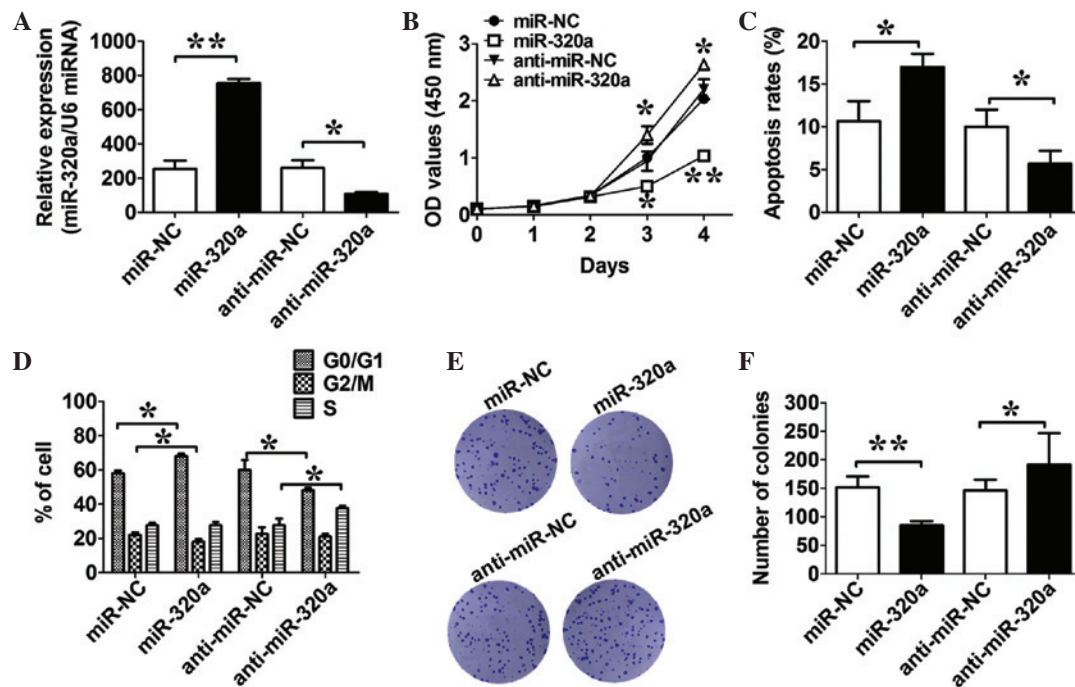


Figure 2. miR-320a suppresses liver cancer cell proliferation *in vitro*. (A) Expression levels of miR-320a were examined by reverse transcription-quantitative polymerase chain reaction in HepG2 cells when transfected with miR-320a or its inhibitors. (B) Analysis of cell proliferation assay (* $P < 0.05$ and ** $P < 0.01$ for miR-320a vs. miR-NC and anti-miR-320a vs. anti-miR-NC). (C) Analysis of cell apoptosis in HepG2 cells when transfected with miR-320a or its inhibitors. (D) Cell cycle analysis of HepG2 cells when transfected with miR-320a or its inhibitors. (E) Colonies formed in HepG2 cells when transfected with miR-320a or its inhibitors. (F) Quantification of the relative colony formation in HepG2 cells when transfected with miR-320a or its inhibitors. Data represent the mean \pm standard error of the mean of 3 replicates. * $P < 0.05$ and ** $P < 0.01$ (one-way analysis of variance followed by Bonferroni's multiple comparison tests). miR, microRNA; NC, negative control.

were estimated by measuring the absorbance at 450 nm using a 96-well plate reader. For the colony formation assay, 5,000 cells were plated in a 6-well plate for 9 days. Colonies were fixed with methanol/acetone (1:1) and stained with crystal violet.

Western blotting. Western blot analysis was performed using anti-cyclin D1 (rabbit monoclonal; 1:4,000 dilution; catalog no. ab137875; Abcam), anti-c-myc (rabbit monoclonal; 1:5,000 dilution; catalog no. ab109416; Abcam), anti-dickkopf-1 (DKK-1; rabbit monoclonal; 1:2,500 dilution; catalog no. ab109416; Abcam) and anti- β -catenin (rabbit polyclonal; 1:3,000 dilution; catalog no. ab6302; Abcam) antibodies, with β -actin (mouse monoclonal; 1:3000 dilution; catalog no. ab20272; Abcam) used as a loading control. The band intensities of the western blotting were analyzed using Image Analysis Software v2.0 (Thermo Fisher Scientific Inc.).

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). The differences among groups were analyzed by a one-way analysis of variance followed by Bonferroni's multiple comparison tests or a t-test, as appropriate. All data are expressed as the mean \pm standard error of the mean. $P < 0.05$ was used to indicate a statistically significant difference.

Results

miR-320a is downregulated in HCC tissues and liver cancer cell lines. The expression level of miR-320a in 35 HCC tissues and their paired adjacent normal liver tissues was quantitatively

A Wild type β -catenin in 3'-UTR: 5'-AAUGUUUUUGCCACAGCUUUU-3'
miR-320a: 5'-AGCGGGAGAGUUGGGUCGAAAA-3'
Mutant β -catenin in 3'-UTR: 5'-AAUGUUUUUGCCGGGAGAAAAU-3'

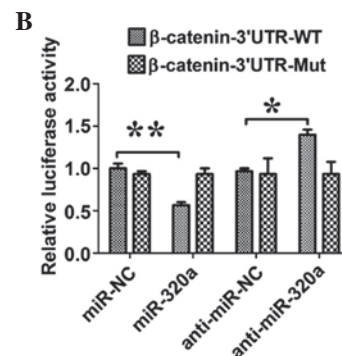


Figure 3. β -catenin is a direct target of miR-320a. (A) Putative miR-320a-binding sequence in the 3'-UTR of β -catenin mRNA. Mutation was generated on the β -catenin 3'-UTR sequence in the complementary site for the seed region of miR-320a, as indicated in bold letters. (B) Analysis of luciferase activity: HEK 293T cells were co-transfected with a control Renilla luciferase expression construct pRL-TK, firefly luciferase reporter plasmid containing either WT or mutant β -catenin 3'-UTR, and either the miR-320a or its inhibitors. Luciferase activity was assayed at 48 h post-transfection. Data represent the mean \pm standard error of the mean of 3 replicates. * $P < 0.05$ and ** $P < 0.01$ (unpaired t-test). miR, microRNA; NC, negative control; WT, wild-type; Mut, mutant; UTR, untranslated region.

analyzed by RT-qPCR. The expression of miR-320a in the HCC tissues was found to be lower than that in the normal tissues ($P = 0.0003$; $n = 35$; Fig. 1A). The expression level of miR-320a

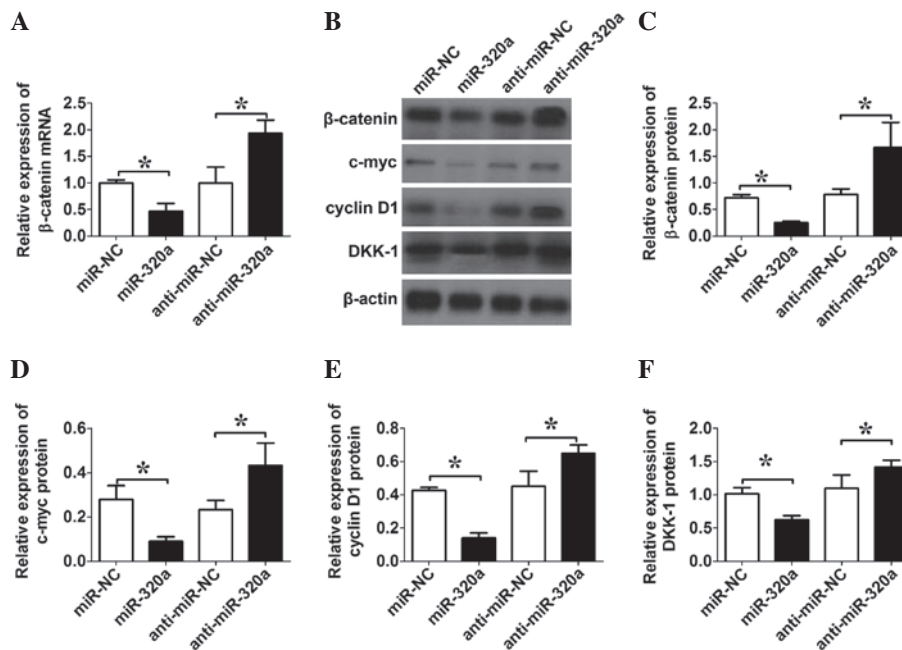


Figure 4. β -catenin is a functional mediator for miR-320a in liver cancer cells. (A) Expression levels of miR-320a were examined by reverse transcription-quantitative polymerase chain reaction in HepG2 cells when transfected with miR-320a or its inhibitors. (B) Western blot analysis of β -catenin, c-myc, cyclin D1 and DKK-1 in HepG2 cells when transfected with miR-320a or its inhibitors. Relative protein expression levels of (C) β -catenin, (D) c-myc, (E) cyclin D1 and (F) DKK-1. * $P < 0.05$ (one-way analysis of variance followed by Bonferroni's multiple comparison tests). miR, microRNA; NC, negative control; DKK-1, dickkopf-1.

was also examined in a panel of human liver cancer cell lines. The results of the RT-qPCR showed that the miR-320a expression level was decreased in all three live cancer cell lines examined compared with the HL-7702 normal liver cells ($P = 0.02$; $n = 3$; Fig. 1B).

miR-320a suppresses liver cancer cell proliferation in vitro.

To evaluate the efficiency of miR-320a and its inhibitors, an RT-qPCR assay was performed to determine the expression level of miR-320a in HepG2 cells transfected with miR-320a or its inhibitors or their relative negative controls. Fig. 2A shows that the level of miR-320a increased significantly following transfection with mimics and that it decreased significantly following transfection with inhibitors (both $P = 0.01$; $n = 3$; Fig. 2A). The role of miR-320a in cell growth was also assessed. As indicated in Fig. 2B, expression of miR-320a in HepG2 markedly inhibited cell growth at day 4 compared with in the HepG2/miR-NC cells. By contrast, expression of anti-miR-320a in HepG2 increased the growth ability when compared to its negative control (both $P = 0.03$; $n = 3$; Fig. 2B). In the apoptosis assay, compared with their respective controls, the expression of miR-320a in the HepG2 cells increased the cell apoptosis rates, and expression of anti-miR-320a in the HepG2 cells decreased the cell apoptosis rates (both $P = 0.03$; $n = 3$; Fig. 2C). The impact of miR-320a on the cell cycle was further assessed. Results showed that the expression of miR-320a increased cell populations at the G_0/G_1 phase, with an associated reduction of cell populations at the G_2/M phase, while the expression of anti-miR-320a caused a reduction of cell populations at the G_0/G_1 phase, with an associated increase of cell populations at the S phase, compared with their respective negative controls (both $P = 0.02$; $n = 3$; Fig. 2D). The colony formation study also showed that the

expression of miR-320a decreased the number of colonies, while the expression of anti-miR-320a increased the number of colonies, compared with their respective negative controls (both $P = 0.01$; $n = 3$; Fig. 2E and F). These results indicated that miR-320a expression suppresses cell proliferation by affecting cell cycle distribution.

β -catenin is a direct target gene of miR-320a in liver cancer cells. To assess miR-320a binding to the 3'-UTR, luciferase reporters were constructed with the β -catenin 3'-UTR. The relative luciferase activity of the construct with WT 3'-UTR was significantly repressed by $\sim 35\%$ following miR-320a transfection, and the expression of anti-miR-320a significantly increased the luciferase activity by $\sim 20\%$, when compared with their respective controls (both $P = 0.03$; $n = 3$; Fig. 3). Site-directed mutagenesis of the miR-320a binding site within the β -catenin 3'-UTR completely abolished the effect of miR-320a or anti-miR-320a transfection.

miR-320a regulates β -catenin-mediated transcriptional activity in liver cancer cells. RT-qPCR was first performed to detect the expression levels of β -catenin mRNA. The expression of miR-320a downregulated the expression levels of β -catenin, while the expression of anti-miR-320a upregulated the expression levels of β -catenin mRNA (both $P = 0.02$; $n = 3$; Fig. 4A). Next, western blotting was performed to detect the expression levels of β -catenin, c-myc, cyclin D1 and DKK-1. The expression of miR-320a in the HepG2 cells downregulated the expression levels of β -catenin, c-myc, cyclin D1 and DKK-1, while the expression of anti-miR-320a upregulated the expression levels of β -catenin, c-myc, cyclin D1 and DKK-1, when compared with their respective negative controls (all $P = 0.04$; Fig. 4B-F).

Discussion

MicroRNAs are small, endogenous non-coding RNAs that are associated with several key biological tumor processes by binding to the 3'-UTRs of targeted genes (5-7). MicroRNAs in HCC progression have been well studied and show an oncogenic or suppressive function (11-13). Previous studies have demonstrated that miR-320a is a novel tumor suppressor that acts by directly targeting the mRNAs of subunit α -1 in HCC, neuropilin 1 and Rac1 in CRC cells, and aquaporin 1 and 4 in cerebral ischemia (15-18). In the present study, it was found that miR-320a was downregulated in HCC tissues and liver cancer cells, and miR-320a exerted its tumor-suppressive function via upregulating the Wnt/ β -catenin signaling pathway.

miR-320a has emerged as a regulator of glycolysis and has been demonstrated to be dysregulated in myasthenia gravis, cerebral ischemia and cancers (15,21,22). In the present study, it was found that miR-320a expression was significantly decreased in HCC tissues and liver cancer cell lines. Functional experiments demonstrated that the overexpression of miR-320a in the HepG2 cells exhibited a marked inhibitory effect on cell proliferation, whereas the expression of anti-miR-320a potentiated the HepG2 cell proliferation. Therefore, all these studies provide evidence that miR-320a is a tumor-suppressive microRNA in HCC.

The present study also showed that miR-320a could induce G₀/G₁ arrest in HepG2 cells, which was further confirmed by results showing that the expression of anti-miR-320a in liver cancer cells reduced the cell population at the G₀/G₁ phase and therefore increased cell growth; this finding is consistent with a previous study in CRC (18). This may at least provide certain insights into the tumor-suppressive mechanism of miR-320a in HCC.

The development and progression of HCC is a complicated process involving stepwise genetic alterations. To understand the functional mechanism of miR-320a as a tumor-suppressive microRNA, bioinformatics analysis was performed to identify the downstream gene targets of miR-320a. β -catenin was demonstrated as one of the newly identified downstream targets of miR-320a in HCC, and β -catenin expression was regulated by miR-320a via direct binding to the 3'-UTR of β -catenin mRNA. This was supported by the result that overexpression of miR-320a suppressed luciferase activity, while expression of anti-miR-320a elevated luciferase expression. The Wnt/ β -catenin signaling pathway plays a central role in the pathogenesis of liver cancer. A large number of studies have revealed the Wnt/ β -catenin cascade as the major driving force behind the proliferative potential of HCC (23-26). Liver cancers almost invariably carry activating mutations in the Wnt/ β -catenin pathway, and the common denominator of the Wnt pathway is the formation of Tcf/ β -catenin complexes, including c-myc and cyclin D1 (27,28). In the present study, it was also observed that the overexpression of miR-320a suppressed the levels of DKK-1, while the expression of anti-miR-320a increased the expression level of DKK-1. Indeed, several studies have reported the overexpression of DKK-1 in HCC tissues and liver cancer cell lines (29-31). In the present study, for the first time, miR-320a was demonstrated to be a negative regulator of β -catenin expression. Previous studies have demonstrated that other microRNAs, including miR-1826

and miR-200a, can also directly bind to the β -catenin 3'-UTR and inhibit its expression in different types of cancer (32-34), suggesting that the microRNA may play an important role in regulating the Wnt/ β -catenin signaling pathway.

In conclusion, the present study demonstrated the function of miR-320a as a growth-suppressive microRNA in human liver cancer, at least partially through the downregulation of β -catenin, which in turn regulates the Wnt/signaling pathway.

Acknowledgements

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