ß-catenin siRNA regulation of apoptosis- and angiogenesis-related gene expression in hepatocellular carcinoma cells: Potential uses for gene therapy

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Abstract. The molecular mechanism responsible for hepatocellular carcinoma (HCC) development remains to be defined although a number of gene pathways have been shown to play an active role, such as Wnt/ß-catenin signaling. In this study, ß-catenin small interfering RNA (siRNA) was designed, synthesized, and transfected into HCC $HepG_2$ cells. RT-PCR and Western blot assays were performed to detect expression of altered genes and proteins, and the MTT assay was used to detect cell viability. Our data showed that ß-catenin mRNA and protein expression levels were effectively knocked down by ß-catenin siRNA and subsequently, tumor cell proliferation was significantly suppressed. Flow cytometry assay showed that tumor cells were arrested at the G_0/G_1 phase of the cell cycles. Molecularly, expression of Smad3, p-caspase-3, and Grp78 protein were upregulated after 72 h of ß-catenin siRNA transfection, whereas expression of TERT, caspase-3, XIAP, MMP-2, MMP-9, VEGF-A, VEGF-C, and bFGF protein were reduced. However, there was no change between the expression of STAT3 and the HSP27 protein following transfection. The results from the current study demonstrated the importance of the Wnt/ß-catenin signaling pathway in regulation of gene expression in HCC. Further studies are required to investigate the role of this pathway in HCC development and targeting of this pathway to control HCC.

Introduction

Hepatocellular carcinoma (HCC) shows high morbidity and is one of the most common malignancies in the world. The prognoses of the patients are very poor due to the late diagnosis and lack of effective treatment. The incidence of HCC varies around the world, for example, HCC is the second leading cause of cancer death in men in China (1,2). Surgery is still the most common method used to treat HCC patients. Chemotherapy is not utilized as often because it is not as effective. Hepatitis B or C virus infection, liver cirrhosis, aflatoxin B1 contamination, and alcohol consumption are the most important risk factors in HCC development. Oncogene activation and tumor-suppressor gene dysfunction are the genetic alterations that contribute to HCC development. To date, a large body of knowledge has been accumulated regarding gene alterations associated with HCC carcinogenesis.

The wnt/ß-catenin signaling pathway plays a key role in development of various cancers, including HCC (3), gastric cancer (4), breast cancer (5), and colon cancer (6). At the molecular level, ß-catenin is a subunit of the cadherin protein complex and has been implicated as an integral component in the Wnt signaling pathway. Recent studies suggest that ß-catenin plays an important role in liver biology, including liver development and regeneration, HGF-induced hepatomegaly, and pathogenesis of liver cancer (7). Wnt plays a specific role in control of ß-catenin function. GSK3 constitutively phosphorylates ß-catenin protein and the phosphorylated ß-catenin is degraded when Wnt protein is not present. However, when Wnt binds to its receptor, Frizzled (Frz) and Dsh will be recruited to the cell membrane inhibiting GSK3 activity; therefore, ß-catenin activity is increased and subsequently translocated into the nucleus to perform a variety of functions, such as abnormal cell growth and eventually cellular carcinogenesis (8). To date, several target genes of this signaling pathway have been identified, including c-jun, c-fos, c-myc, cyclin D_1 (9), survivin (10), and PPARγ. Even so, recently, STAT3 is reported to be involved in the nuclear accumulation of ß-catenin in colorectal cancer (11), and Wnt/ß-catenin pathway upregulated levels of STAT3 mRNA and protein in esophageal cancer cells (12). Smad3 signaling in proximal tubular epithelial cells is augmented by the inhibition of ß-catenin expression (13). ß-catenin expression is related to the upregulation of hTERT in gallbladder carcinoma. Furthermore, caspase-3-dependent cleavage

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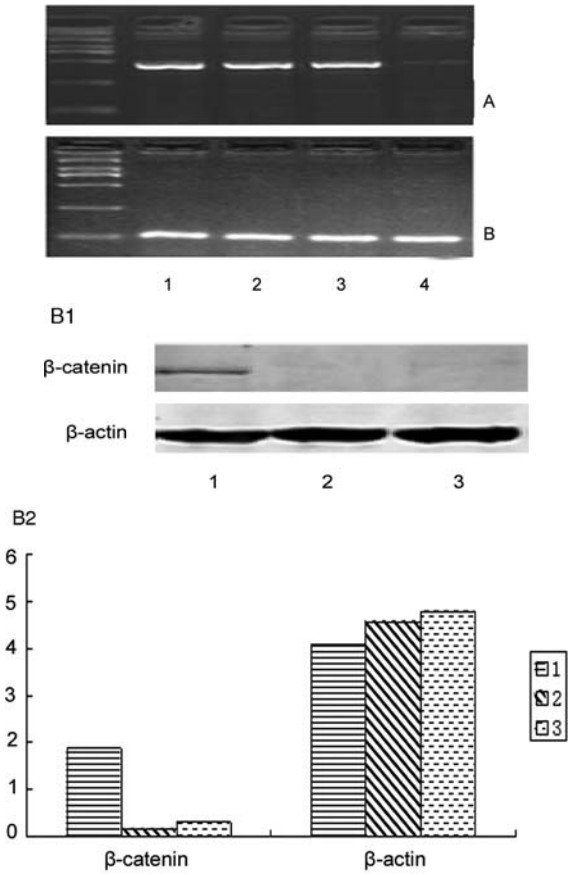

Figure 1. Knockdown of ß-catenin expression. (A) RT-PCR. HCC $HepG_2$ cells were grown and transiently transfected without any siRNA-Lipofectamine 2000 (1), or with Lipofectamine 2000 only (2), random siRNA oligonucleotides (3), or ß-catenin siRNA (4). RNA from the cells were extracted and subjected to RT-PCR analysis of ß-catenin and ß-actin expression. (B) Western blotting. HCC $HepG_2$ cells were grown and transiently transfected with random siRNA oligonucleotides or ß-catenin siRNA for 72 and 96 h. The transfected cells were subjected to protein isolation and Western blot analysis of ß-catenin expression. (B1) 1, The control group; 2, 72 h after the transfection; 3, 96 h after the transfection. (B2) (x-axis) 1, the control group; 2, 72 h after the transfection. y-axis is the quantitative results of gray-scale analysis.

of ß-catenin was demonstrated to occur during apoptosis (14) and in NF-κB inhibitor-induced XIAP and ß-catenin mRNA levels in HCC cells: $HepG_2$, HA22T/VGH and HuH-6 (15). Other studies have demonstrated that the ß-catenin pathway upregulated expression of VEGF and MMP-7 in human colorectal cancer (16).

The current study aimed to silence ß-catenin expression using RNA interference (RNAi) technology in HCC cells and investigated phenotypic and molecular changes, which may in turn be helpful in development of a novel strategy to control HCC.

Materials and methods

Materials. The HCC cell line, $HepG_2$, was obtained from the Cell Bank at Chinese Academy of Sciences (Shanghai, China). Lipofectamine™ 2000 was from Invitrogen (Carlsbad, CA, USA). The Shanghai GenePharma Co. (Shanghai, China) designed and synthesized ß-catenin siRNA. The siRNA sequences were: 5'-GGGUUCAGAUGAUAUAAAUTT-3' (sense); and 5'-AUUUAUAUCAUCUGAACCCAG-3' (antisense). Dulbecco's minimum essential medium (DMEM) with high glucose was purchased from HyClone Corp. (Waltham, MA, USA) and fetal calf serum (FCS) was from the TBD Biotechnology Corp. (Tianjin, China). Santa Cruz Biotechnology (Santa Cruz, CA, USA) provided the following antibodies: mouse anti-ß-catenin, anti-Hsp-27, anti-VEGF-A, anti-bFGF, and anti-ß-actin; rabbit anti-STAT3, anti-Smad3, anti-TERT, anti-MMP-2, and anti-MMP-9; and goat anti-VEGF-C and anti-Grp-78. Rabbit anti-caspase-3 and anti-p-caspase-3 and goat anti-XIAP antibodies were from Cell Signaling Technology (Danvers, MA, USA).

Cell culture. $HepG_2$ cells were plated in culture flasks and cultured in DMEM supplemented with 10% v/v FCS at 37˚C in a humidified 95% air and 5% carbon dioxide atmosphere. The cells were subcultured when a confluence was achieved by 0.25% trypsin digestion.

Transient gene transfection. A number of $3x10^5$ cells were plated in 6-well plates in triplicate and grown to ~30-50% confluence. siRNA in Lipofectamine 2000 plus 2 ml of DMEM without FCS were applied to each well for gene transfection. After 6 h of transfection, the media were replaced with DMEM containing 10% v/v FCS. The cells were harvested at either 72 or 96 h, and mRNA and protein were isolated from the cells for analyses of gene expression. The cells without any gene transfection, Lipofectamine 2000 without siRNA, and a random siRNA oligonucleotide transfection were the three controls used in this study. All experiments were performed in triplicate and representative results are reported.

Methyl thiazolyl tetrazolium (MTT) assay. The tumor cells were seeded in 96-well plates in triplicate and transfected with ß-catenin siRNA vector or control vector using Lipofectamine 2000. After 72 h, 20 μl (5 mg/ml) of MTT diluted in PBS was added into the growth medium and incubated for 4 h. The cells were then dissolved with 200 μl of dimethyl sulphoxide and a microplate reader (Model MK3, Thermo Labsystems Co., USA) was used to measure the optical density at a single wavelength of 540 nm. The percentage of cell viability was calculated using the following formula: % control = ODt/ODc x 100, where ODt and ODc are the optical densities for treated and control cells, respectively.

Flow cytometry assay. The tumor cells were grown and transfected with either ß-catenin siRNA vector or control vector, and 72 h later, the cells were trypsinized and fixed with 2 ml of ice-cold 70% ethanol and preserved at 4˚C. The cells were then washed and a cell cycle kit (BD Corp., USA) was used to remove the RNases and proteins. After that, the cells were incubated in 10 g/ml propidium iodide (PI) at 4˚C for 10 min in the dark and subjected to cell cycle analysis using a CyFlow ML flow cytometer (Partec Co., Germany).

Reverse transcription polymerase chain reaction (RT-PCR). The cells were grown in monolayer and transfected with either ß-catenin siRNA vector or control vector, and 72 h later, RNA from the cells was isolated using TRIzol reagent (Invitrogen) and subjected to RT-PCR analysis of gene expression. Briefly, RNA was first converted to cDNA using

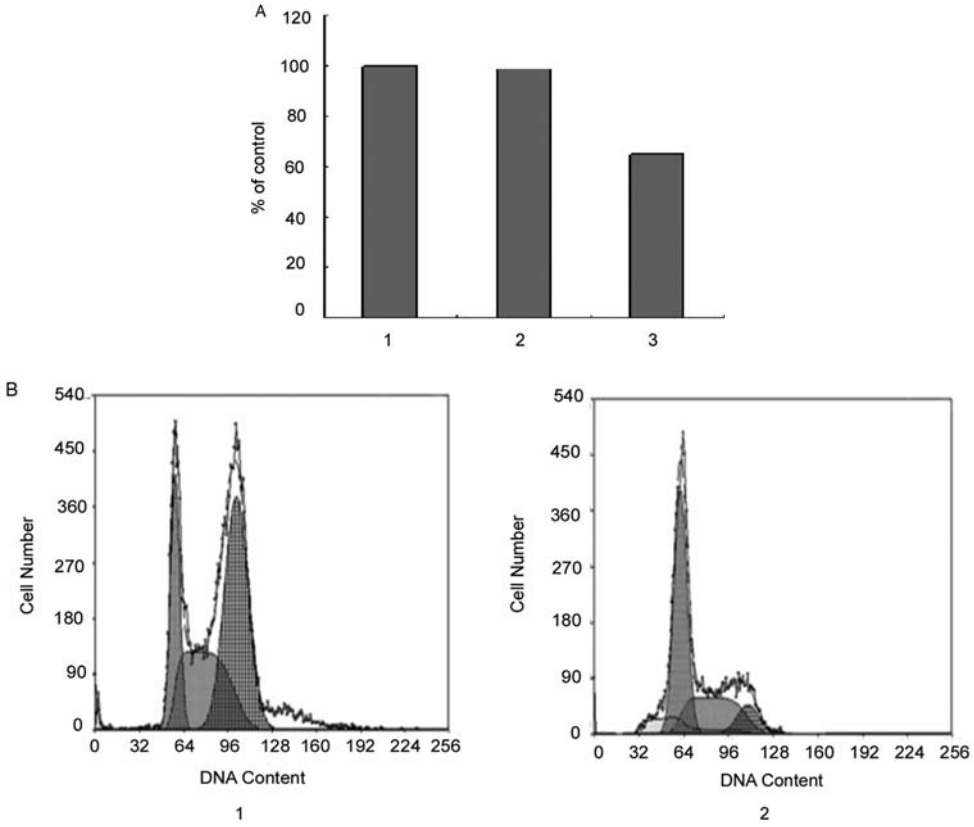

Figure 2. Reduction of tumor cell viability and the G_0/G_1 cell cycle arrest after knockdown of ß-catenin expression. (A) MTT assay. HCC $HepG_2$ cells were grown in 96-well plates in triplicate and transiently transfected with random siRNA oligonucleotides or ß-catenin siRNA for 72 h. A MTT assay was preformed to detect the change in cell viability. The percentage of cell growth was calculated using the formula: % of control = ODt/ODc x 100, where ODt and ODc are the optical densities for treated and control cells, respectively. 1, control group; 2, Lipofectamine 2000; 3, siRNA + Lipofectamine 2000. (B) Flow cytometry assay. The cells were grown and transiently transfected with random siRNA oligonucleotides (1) or ß-catenin siRNA for 72 h (2) and then subjected to a flow cytometry assay.

a reverse transcription kit (Promega Corp., Madison, WI, USA), and the cDNA was subjected to PCR amplification of ß-catenin and ß-actin with a PCR kit (Tiangen Biotechnology Co., Tiangen, China). The primer sequences were: ß-catenin, 5'-AGGAAGGGATGGAAGGTCTC-3' and 5'-CGCTGGGT ATCCTGATGTGC-3', which generated a 462 bp band; ß-actin, 5'-CCCAGCACAATGAAGATCAAGATCAT-3' and 5'-ATCTGCTGGAAGGTGGACAGCGA-3', which generated a 101 bp band. The PCR conditions were: for ß-catenin, 94˚C for 3 min, 94˚C for 30 sec, 57˚C for 30 sec, 72˚C for 1 min for 30 cycles and 72˚C for 5 min. The conditions for ß-actin were: 94˚C for 3 min, 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min for 30 cycles and 72˚C for 5 min. PCR products were separated on 2% agarose gels, photographed, and analyzed using a gel imaging system.

Protein isolation and Western blotting. The cells were grown and transfected either with or without siRNA for 72 and 96 h and total cellular protein was isolated in RIPA buffer (Solarbio Technology Co., Beijing, China). The protein concentrations were determined using a BCA kit (Beyotime Biotechnology Co., Shanghai, China). Protein samples were electrophoresed on 10-15% SDS-PAGE gels and transferred onto PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were then incubated in PBS containing 5% skim milk at 37˚C for 1 h, and with specific primary antibodies at 4˚C overnight. The next day, the membranes were washed 3 times with PBS, followed by incubation with HRP-conjugated secondary antibodies (1:5000 dilution) at 37˚C for 1 h. After a second wash of 3 times with PBS, the target proteins were developed in 3,3'-diaminobenzidine (DAB). Once protein bands were visible on the membranes, color development was stopped and the membranes were rinsed in distilled water to capture images using Gel Imaging System. Quantity One software was used to analyze each of the images. The quantitative results from the gray-scale analysis were used for the primary statistical analyses.

Statistical analysis. The data were analyzed using Student's t-test. $P<0.05$ was considered to be statistically significant.

Results

Knockdown of ß-catenin expression. We first determined the ability of ß-catenin siRNA to knock down ß-catenin expression. After gene transfection, RT-PCR showed that expression of ß-catenin mRNA is reduced significantly compared to the ß-actin reference ($t=14.33$, $P<0.01$). All three controls (i.e., the cells without any gene transfection, Lipofectamine 2000 without siRNA, and a random siRNA oligonucleotide transfection) showed no changes in ß-catenin mRNA levels ($F=0.99$, $P>0.05$). Furthermore, ß-catenin protein was also decreased at both 72 and 96 h after gene transfection ($t=4.43$, $P<0.05$) (Fig. 1).

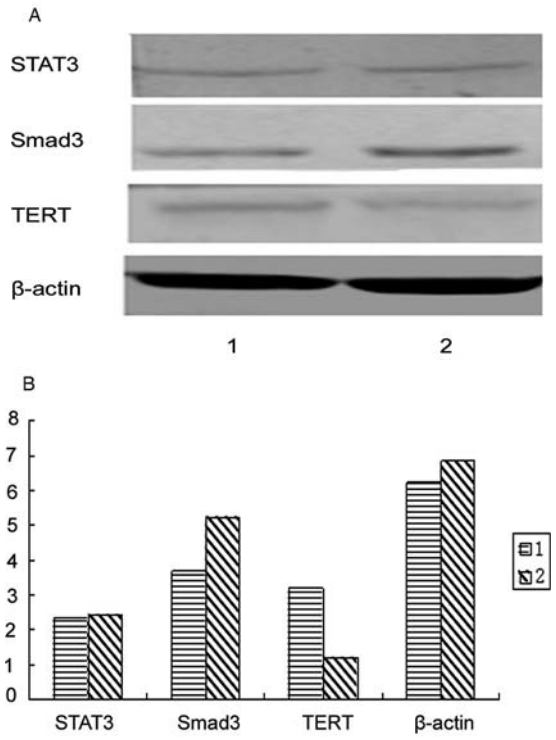

Figure 3. Expression of STAT3, Smad3, TERT, and ß-actin proteins after knockdown of ß-catenin expression. (A) 1, The control group; 2, 72 h after the transfection. (B) (x-axis) 1, the control group; 2, 72 h after the transfection. y-axis is the quantitative results of gray-scale analysis.

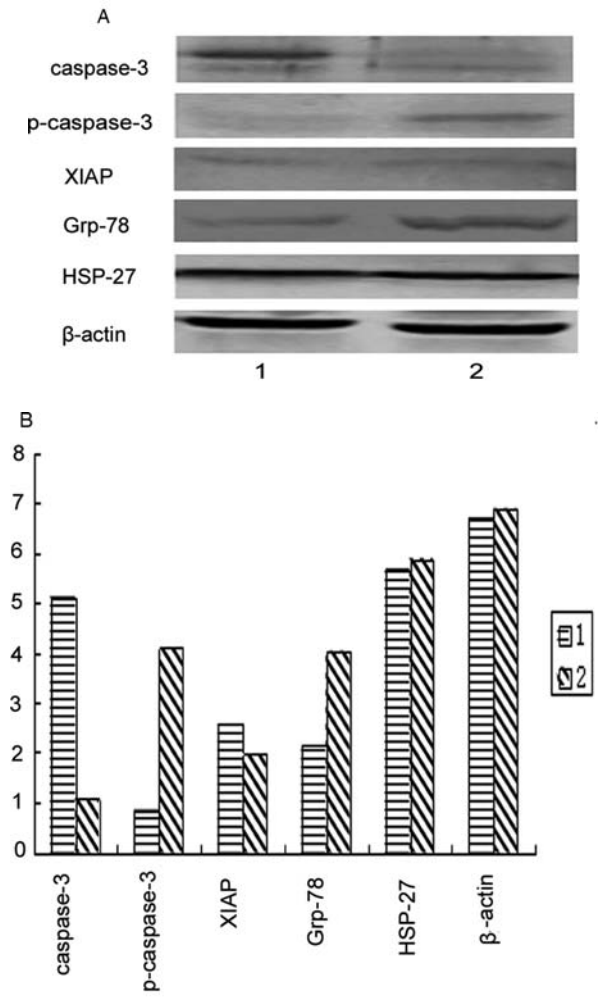

Figure 4. Expression of caspase-3, p-caspase-3, XIAP, Grp-78, HSP-27, and ß-actin proteins after knockdown of ß-catenin expression. (A) 1, The control group; 2, 72 h after the transfection. (B) (x-axis) 1, the control group; 2, 72 h after the transfection. y-axis is the quantitative results of gray-scale analysis.

Reduced tumor cell viability after knockdown of ß-catenin expression. We then assessed the phenotypic changes after knockdown of ß-catenin expression, and found that transfection of ß-catenin siRNA significantly inhibited tumor cell proliferation using the MTT assay. Specifically, ß-catenin siRNA reduced tumor cell viability by 30.0% after 72 h of gene transfection ($q=8.12$, $P<0.01$) (Fig. 2). Upon 72 h ß-catenin siRNA transfection, flow cytometry assay further proved that tumor cells were arrested at the G_0/G_1 phase of the cell cycles (55 vs. 20% of the control cells) ($\chi^2=47.739$, $P<0.01$) (Fig. 2).

Modulation of protein expression after knockdown of ß-catenin expression. To further understand regulation of the downstream genes by ß-catenin, we detected various protein expressions using Western blot analysis. Our data presented that there were no changes in expression of the STAT3 protein after ß-catenin siRNA transfection ($F=0.49$, $P>0.05$), whereas Smad3 protein was increased at 72 h ($t=10.67$, $P<0.05$). In contrast, TERT protein expression was reduced at 72 h ($t=4.18$, $P<0.05$) (Fig. 3). ß-catenin siRNA transfection also altered apoptosis-related genes (such as Grp-78, caspase-3, XIAP, and HSP-27). The p-caspase-3 protein was increased 72 h after ß-catenin siRNA transfection, whereas the caspase-3 protein was reduced. The expression of the XIAP protein was decreased at 72 h ($t=6.87$, $P<0.05$), while the Grp78 protein was increased at 72 h ($t=4.18$, $P<0.05$). However, there was no change in gene expression of the HSP27 protein upon gene transfection ($F=1.91$, $P>0.05$) (Fig. 4).

Modulation of angiogenesis factor expressions by ß-catenin siRNA. We then determined the role of ß-catenin siRNA in the regulation of angiogenesis factor expressions and found that expression of the MMP-2 and MMP-9 proteins were reduced 72 h after ß-catenin siRNA transfection. In addition, expression of VEGF-A and VEGF-C was also inhibited 72 h after gene transfection ($t=10.36$ and $t=15.31$, $P<0.01$). The bFGF expression showed a pattern that was similar to those observed for VEGF and MMP, with inhibition ($t=44.11$, $P<0.01$) (Figs. 5 and 6).

Discussion

RNAi, first reported by Fire *et al* in 1998, is a novel gene silencing technique where RNAi can specifically degrade homologous mRNA after introduction of a double-stranded RNAi into cells. Therefore, regulation of the target gene expression occurs at the post-transcriptional level (17). The study of gene function has successfully implemented the application of RNAi as a powerful tool (18). The knockdown of oncogene expression by RNAi was tested and applied to

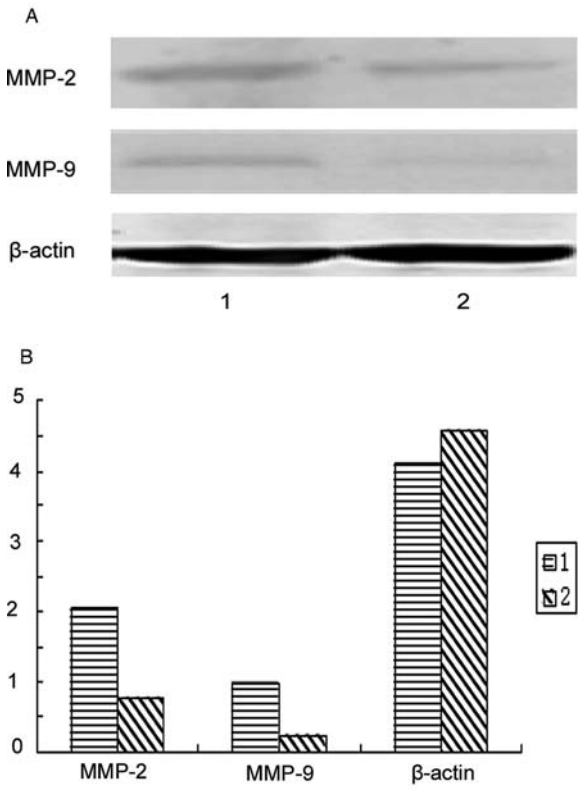

Figure 5. Expression of MMP-2, MMP-9, and ß-actin proteins after knockdown of ß-catenin expression. (A) 1, The control group; 2, 72 h after the transfection. (B) (x-axis) 1, the control group; 2, 72 h after the transfection. y-axis is the quantitative results of gray-scale analysis.

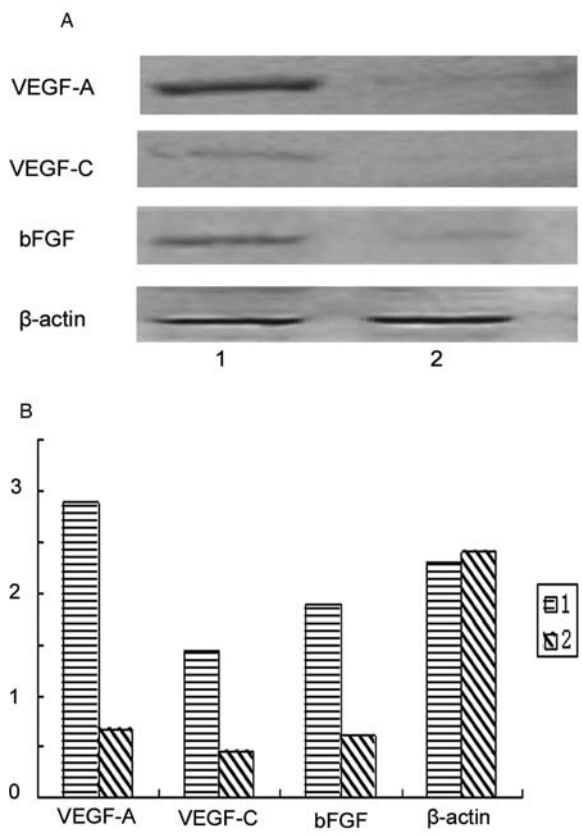

Figure 6. Expression of VEGF-A, VEGF-C, bFGF, and ß-actin proteins after knockdown of ß-catenin expression. (A) 1, The control group; 2, 72 h after the transfection. (B) (x-axis) 1, the control group; 2, 72 h after the transfection. y-axis is the quantitative results of gray-scale analysis.

tumor therapies, eventually showing some success (19-21). Our current study demonstrates the successfully suppressed expression of ß-catenin mRNA 24 h after gene transfection. The study also shows suppression of ß-catenin protein 72 and 96 h after ß-catenin siRNA transfection in HCC $HepG_2$ cells. Previous studies demonstrated that Wnt/ß-catenin signaling pathways play particularly important roles in the liver (7). Alteration of this molecular pathway may lead to HCC development (7), and Wnt/ß-catenin signaling is involved in number of different gene expressions, such as cell growth and apoptosis-related genes.

Indeed, our study demonstrated that ß-catenin siRNA transfection reduced tumor cell viability and arrested the cells at the G_0/G_1 phase of the cell cycles. Further progression showed that ß-catenin siRNA transfection regulated expression of apoptosis-related genes, such as caspase-3 and XIAP proteins. Apoptosis is primarily regulated by caspases and inhibitors of the apoptosis protein (IAP) (22). Caspase-3 plays a critical role in the execution of apoptosis, and its abnormal expression and activation have been demonstrated in many tumors. Nevertheless, IAP family members are the known endogenous caspase inhibitors. The X linked inhibitor of the apoptosis protein (XIAP) is an important inhibitor (23). Abnormal expression of XIAP has been demonstrated in many tumors, including HCC (24,25) and third grade ductal carcinomas (26). Our findings showed that the expression of caspase-3 and XIAP proteins was reduced 72 h after ß-catenin suppression, whereas the p-caspase-3 protein was increased, resulting in an induction of tumor cell apoptosis, thereby reducing cell viability. These data indicated that XIAP and caspase-3 are the downstream targets of the Wnt/ß-catenin signaling pathway.

Cell adhesion and angiogenesis factors play an important role in tumor progression and metastasis (27-29). To date, a number of these factors have been discovered, such as VEGF (27), TF, MMP (28), FGF, and TNF (30). MMPs promote angiogenesis and contribute to tumor infiltration and metastasis not only through degradation of the extracellular matrix and vascular basilemma, but also through the active regulation of transforming growth factor ß (TGF-ß), bFGF, and VEGF (31). The formation of blood and lymphatic vessels, vascular permeability, and endothelial cell survival is regulated by the VEGF family (30). The expression of bFGF has been correlated with the promotion of cancer cell proliferation and tumor angiogenesis (32). bFGF also regulates activities of collagenase, protease, urokinase-type plasminogen activator (uPA), and integrins. Our current data showed that knockdown of ß-catenin expression in HCC cells decreased expression of MMP-2, MMP-9, VEGF-A, VEGF-C, and bFGF, indicating that the Wnt/ß-catenin signaling pathway plays a role in HCC angiogenesis. However, the underlying mechanism of this regulation remains to be determined. A previous study indicated that further investigation was worthwhile by showing that the Wnt/ß-catenin signaling pathway acted directly on the MMP gene

promoter through LEF1/TCF binding in T cells (33), but this regulatory mechanism has not been reported in HCC.

Furthermore, STAT3 is a key molecule in the JNK/Stat pathway and its activation has shown an oncogenic activity (34). However, in the present study, we did not find any changes in STAT3 protein levels after inhibition of the ß-catenin protein, suggesting that STAT3 does not participate in the ß-catenin-regulated HCC cell growth and apoptosis. This result is in contrast to previous reports that ß-catenin upregulated STAT3 expression in esophageal cancer (12), colon cancer, and embryonic stem cells (35). These conflicting data may reflect a difference among different tumors.

Lastly, Smad is a part of the TGF-ß pathway and the key factor in regulation of TGF-ß action (36). TERT is the catalytic subunit of telomerase and a major limiting factor of telomerase activation (37). Our current data demonstrated that inhibition of ß-catenin expression induced the expression of Smad3 protein, but reduced TERT protein expression. Another previous study presents similarity to our data such that the inhibition of ß-catenin augmented Smad3 signaling in proximal tubular epithelial cells (13). Smad3 has been know to block telomerase activation as shown in recent studies (38). By what mechanism ß-catenin regulates these gene expressions warrants further investigation.

In summary, the Wnt/ß-catenin signaling pathway regulates the expression of several other signaling pathway genes, such as Smad3, TERT, caspase-3, XIAP, Grp-78, MMP-2, MMP-9, VEGF-A, VEGF-C, and bFGF, thereby participating in HCC development. However, ß-catenin signaling does not alter the expression of STAT3 and HSP27 proteins in HCC cells. The verified data will help us better understand HCC pathogenesis and the role of the Wnt/ß-catenin signaling pathway in HCC development, and may in turn be used for gene therapy of HCC.

Acknowledgements

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