doi:10.4149/neo_2011_03_239

Wnt/β-catenin signaling regulates MAPK and Akt1 expression and growth of hepatocellular carcinoma cells

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Received August 30, 2010

In hepatocellular carcinoma (HCC), Wnt/β-catenin, Ras/MAPK and PI3K/AKT signaling pathways form a complex network and play important roles during HCC genesis and development. To study their relationship and the influence on cell growth, the siRNA directed against β-catenin was transfected into HCC $HepG_2$ cells. β-catenin mRNA and protein levels were measured respectively at various times by RT-PCR and Western blot. Furthermore, HCC cell growth was measured by MTT assay. Finally, MAPK family and Akt1 protein levels were also measured by Western blot. After the transfection, β-catenin mRNA levels were markedly inhibited at 24 h and increased gradually at 48, 72 and 96 h; β-catenin protein levels decreased gradually at 24, 48 and 72 h and slightly increased at 96 h. HCC cell growth was inhibited from 24–72 h, but this inhibition decreased at 96 h. ERK1/2 (p42/p44 MAPK), JNK/SAPK, p38 MAPK, and Akt1 protein levels showed no change following transfection, while their phosphorylated protein levels showed changes. Thus, siRNA directed against β-catenin markedly decreased β-catenin gene expression and inhibited cell growth. Wnt/β-catenin signaling pathway might regulate Ras/MAPK and PI3K/Akt signaling pathways through regulation of the phosphorylation state of ERK1/2, JNK/SAPK and Akt1 protein in HCC $HepG_2$ cells. These pathways might compensate for the inhibitory effect of β-catenin, thereby affecting tumor cell growth and others downstream factors.

Key words: hepatocellular carcinoma; β-catenin; MAPK; AKT1; signaling pathway

Hepatocellular carcinoma (HCC) is one of the most common human malignancies. Previous work has demonstrated that many signaling pathways play key roles during the pathogenesis of HCC. These pathways include Wnt/β-catenin, Ras/MAPK, PI3K/Akt, JNK/Stat, NF-κB, TGF-β/Smad, Hh, and p53 pathways, in which Wnt/β-catenin, Ras/MAPK, and PI3K/Akt are particularly important and interrelated.

The cytoplasmic protein β-catenin is a central molecule in Wnt signaling pathway [1] and Wnt/β-catenin signaling has been shown to be deregulated in HCC [2,3], gastric cancer [4], breast cancer [5], and colon cancer [6]. When β-catenin phosphorylation and ubiquitin-dependent degradation are inhibited, β-catenin concentrates in the cytoplasm, thereby resulting in abnormal cell growth and eventually carcinogenesis [7].

Mitogen-activated protein kinase (MAPK) and Akt, also called protein kinase B (PKB), are both serine/threonine protein kinases. They participate in the regulation of tumor cell growth, differentiation, proliferation, apoptosis, angiogenesis, tumor metastasis, and cell cycle [8,9]. At present, four MAPK pathways have been identified in mammalian cells, including the extracellular signal regulated protein kinase (ERK), c-Jun amino-terminal kinase (JNK)/stress activated protein kinases (SAPK), p38MAPK, and big MAP kinase 1(BMK1)/ERK5 pathway. Akt family members include Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ.The pathogenesis of many tumors in humans is related to the abnormal expression of MAPK and Akt, including gastric cancer [10,11], liver cancer [12,13], colon cancer [14], and lung cancer [15], as well as others [16].

Previous research indicates that Akt might inhibit β-catenin degradation and enhance its stability [17]. The PI3K/Akt signaling pathway plays a crucial role during the development of human embryonic stem cells in the anterior primitive streak (PS), in part, by enhancing β-catenin stability [18]. P-MAPK expression was shown to be strongly dependent on β-catenin during early Xenopus development [19], and activation of

β-catenin signaling provides a potential mechanism for p38 MAPK-mediated survival in specific tissues [20]. Together, these data indicate that β-catenin is related to the expression of MAPK and Akt, but their exact relationship is still unclear in HCC.

Considering the above observations, we silenced β-catenin using RNA interference (RNAi) and estimated cell growth and the expression of MAPK and Akt1 protein in the HCC $HepG_2$ cell line in order to further elucidate the pathogenesis of HCC and its relationship to Wnt/β-catenin and Ras / MAPK, PI3K/Akt pathways.

Materials and methods

Cell culture. $HepG_2$ cells, acquired from the cell bank of the Chinese Academy of Sciences, were plated in culture flasks and cultured in Dulbecco's minimum essential medium (DMEM, HyClone Corporation, Waltham, MA, USA) supplemented with 10% vol/vol fresh fetal calf serum (FCS) (TBD Biotechnology Corporation, Tianjin, China) at 37°C in a humidified, 5% carbon dioxide atmosphere. Cells were replated following a 1 min 0.25% trypsin digestion when they reached confluency in order to maintain the cell line.

Transient transfection. SiRNA directed against β-catenin was designed and synthesized by Shanghai Genepharma Company. The effective siRNA sequences were: sense, 5'- GGGUUCAGAUGAUAUAAAUTT -3'; and antisense, 5'- AUUUAUAUCAUCUGAACCCAG -3'. Negative control siRNA sequences were: sense 5'- UUCUCCGAACGUGUCACGUTT -3'; antisense 5'- ACGUGACACGUUCGGAGAATT -3'. GAPDH positive control siRNA sequences were: sense 5'- GUAUGACAACAGCCUCAAGTT -3'; antisense 5'- CUUGAGGCUGUUGUCAUACTT -3'.

A total of 3×10^5 cells were plated in six-well plates in triplicate and grown to approximately 30-50% confluency. For transfection, 500 μl of siRNA in Lipofectamine™ 2000 (Invitrogen) was applied to each well containing cells and 2 ml of DMEM media without FCS, and the plates were gently rocked back and forth. At 6 h after the transfection, the media was replaced with DMEM media containing 10% vol/vol FCS. The cells were harvested at 72 and 96 h, and mRNA and protein were isolated for later analyses. Control groups without the addition of transfection reagents were also analyzed. All experiments were performed in triplicate and representative results were reported.

Detection of β-catenin mRNA expression by RT-PCR. Obtained specimens were subdivided into the following groups: (1) control without siRNA-Lipofectamine™ 2000 complexes; (2) Lipofectamine™2000 transfection without siRNA; (3) negative control siRNA transfection; (4) GAPDH positive control siRNA transfection; and (5) Lipofectamine™2000 siRNA transfection.

The siRNA was transfected into $HepG_2$ cells (as described above), and then total RNA from groups 1–4 was extracted at 24 h, and total RNA from group 5 was extracted at 24, 48, 72, and 96 h. Total RNA was isolated from each cell population using Trizol reagent (Invitrogen Corporation), performed according to the manufacturer's instructions, and concentrations were measured by spectrophotometer [A260/A280=2.2]. RNA was converted into double-stranded cDNA using a reverse transcription kit (Promega Corporation), and concentrations were measured by spectrophotometer. Subsequent PCR amplifications of targeted β-catenin, β-actin reference, and a GAPDH positive control were performed with cDNA and primers using a PCR kit (Tiangen Biotechnology Co.).The primer sequences and the expected lengths of amplification fragments are listed as follows. β-catenin upstream primer: 5'- AGGAAGGGATGGAAGGTCTC -3'; downstream primer: 5'- CGCTGGGTATCCTGATGTGC -3'; 462 bp. β-actin upstream primer: 5'- CCCAGCACAATGAAGATCAAGATCAT -3'; downstream primer: 5'- ATCTGCTGGAAGGTGGACAGCGA -3'; 101 bp. GAPDH upstream primer: 5'- GGTGAAGGTCGGTGTGAACGGATTT -3'; downstream primer: 5'- AATGCCAAAGTTGTCATGGATGACC -3'; 502 bp. The PCR conditions were mentioned as follows. β-catenin: 94°C 3 min; 94°C 30 s, 57°C 30 s, 72°C 1 min, 30 cycles; 72°C 5 min. β-actin: 94°C 3 min; 94°C 30 s, 55°C 30 s, 72°C 1 min, 30 cycles; 72°C 5 min. GAPDH: 94°C 3 min; 94°C 30 s, 55°C 30 s, 72°C 1 min, 30 cycles; 72°C 5 min. PCR products were separated on 2% agarose gels and photographed and analyzed using a gel imaging system.

Detection of β-catenin protein expression by Western blot. Transfected cells were lysed in RIPA buffer at 24, 48, 72, and 96 h post-transfection for protein isolation. Protein concentrations were determined using the BCA kit (Beyotime Biotechnology Co., Shanghai, China). Protein samples were electrophoresed on 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore Corporate, Billerica, MA, USA). The membranes were blocked by incubation in PBS containing 5% skim milk at 37°C for 1 h, and then were incubated with primary antibodies against β-catenin and β-actin (1:200 dilution, Santa Cruz Biotechnology) at 4°C overnight. The membranes were rinsed 3 times with PBS, followed by incubation with HRP-conjugated secondary antibodies (1:5000 dilution) at 37°C for 1 h. After rinsing 3 times with PBS, the membranes were developed in 3, 3'-diaminobenzidine (DAB) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) When protein bands were visible on the membranes, color development was discontinued and membranes were rinsed in distilled water. Images were captured using a gel imaging system and were analyzed using Quantity One software [Source, location]. The quantitative results of gray-scale analysis were used for statistical analysis.

Detection of HCC cell growth by methyl thiazolyl tetrazolium (MTT) assay. A total of 1.5×10^4 transfected cells and controls were plated in 96-well plates in triplicate, which were grown to about 30-50% confluence at the time of transfection. A total of 50 μl of siRNA-Lipofectamine™ 2000 complexes was applied to each well containing cells and 100 μl of DMEM medium without FCS, followed by gentle rocking. Medium

was replaced by DMEM medium containing 10% vol/vol FCS at 6 h after the transfection. A total of 20 μl (5 mg/ml) of MTT diluted in PBS was added to medium at 24, 48, 72, and 96 h after the transfection. After 4 h incubation, medium was removed and the sediments were left at the bottom of wells. Dimethyl sulphoxide (DMSO, 200 μl) was applied to each well and the sediments were redissolved by rocking the plate. Finally, the absorbance was measured using a microplate reader at 540 nm to determine the number of viable cells [21]. All experiments were performed in triplicate. The data were normalized to their respective controls.

MAPK and Akt protein levels by Western blot. Transfected and control cells were collected at 72 and 96 h. MAPK and Akt protein levels was measured by Western blot, as described for β-catenin protein, with the exception of the corresponding primary and secondary antibodies used (Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution).

Statistical analysis. Data were compared using the Student's *t*-test and X^2 test. $P < 0.05$ was considered to be statistically significant.

Results

β-catenin mRNA levels. β-catenin mRNA levels of the control group showed no significant difference from that of the Lipofectamine™ 2000 transfection group or the negative control siRNA transfection group at 24 h ($F = 0.99$, $P > 0.05$, while a significant decrease in β-catenin mRNA expression was observed in the siRNA transfection group ($t = 14.33$, $P < 0.01$). These observations also showed the efficacy of the siRNA. The mRNA levels of the GAPDH positive control group decreased significantly at 24 h ($t = 254.39$, $P < 0.01$), indicating that the transfection procedure was effective. After siRNA transfection, β-catenin mRNA level was markedly decreased at 24 h and gradually increased further at 48, 72, and 96 h; however, the level at 96 h was still below that of the control group (see Figure 1). These results indicated that the siRNA transfection could specifically silence β-catenin mRNA levels.

β-catenin protein expression. After siRNA transfection, β-catenin protein levels were detected by Western blot at 24, 48, 72, and 96 h. The expression decreased gradually at 24, 48, and 72 h, but at 96 h it showed a slight increase in comparison with that at 72 h ($t = 5.55$, $P < 0.01$) (see Figure 2). According to the time-dependent expression of β-catenin protein, we determined that 72 and 96 h would be the optimal time points for detecting the levels of other signaling proteins.

HCC cell growth by MTT assay. The results of the MTT assays are listed in Figure 3. Cell viability in the control group showed no significant difference relative to that of the Lipofectamine™ 2000 only group, $q = 0.84$, $P > 0.05$; however, the siRNA transfection group showed a significant decrease in the number of viable cells, $q = 8.12$, $P < 0.01$. Cell growth inhibition ratios induced by the siRNA were 21.1% at 24 h, 29.0% at 48 h, 30.0% at 72 h, and 20.5% at 96 h. These results indicated that HCC cell growth was inhibited or cell death was

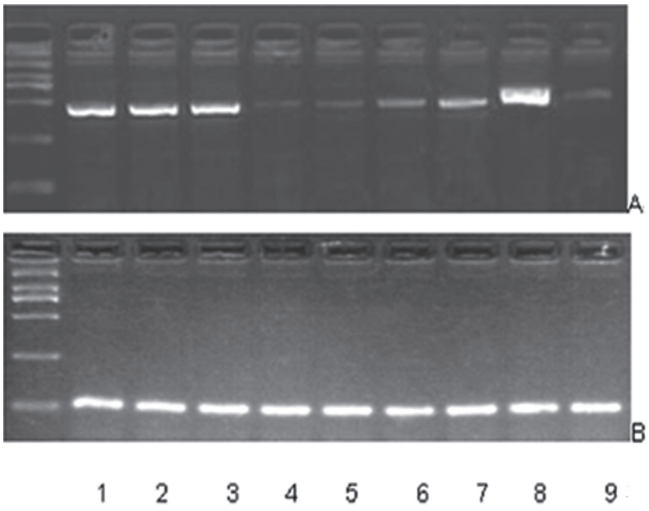

Figure 1. mRNA levels of β-catenin, GAPDH and β-actin. Cells were plated and grown to approximately 30-50% confluency. For transfection, 500 μl of siRNA in Lipofectamine™ 2000 (Invitrogen) was applied to each well containing cells and DMEM media without FCS. At 6 h, the media was replaced with DMEM media containing 10% vol/vol FCS. The cells were harvested at 72 and 96 h. Control groups without the addition of transfection reagents were also analyzed. All experiments were performed in triplicate and representative results were reported. A, β-catenin mRNA expression of groups 1-7 and GAPDH mRNA expression of groups 8-9; B, β-actin mRNA expression. 1, Control without siRNA-Lipofectamine™ 2000 complexes; 2, Lipofectamine™2000 transfection without siRNA.; 3, Negative control siRNA transfection; 4, The siRNA transfection for 24 h; 5, The siRNA transfection group for 48 h; 6, T siRNA transfection for 72 h; 7, siRNA transfection for 96 h; 8, GAPDH control without siRNA-Lipofectamine™ 2000 complexes; 9, GAPDH positive control siRNA transfection.

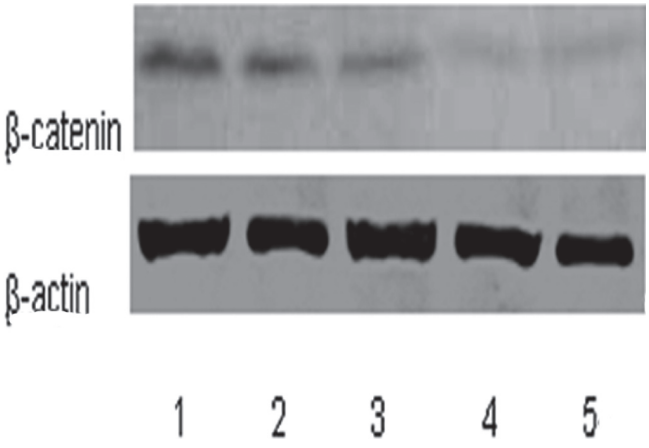

Figure 2. Protein levels of β-catenin and β-actin. Transfected cells were lysed at 24, 48, 72, and 96 h post-transfection. Protein concentrations were determined using a BCA kit. Protein samples were electrophoresed on 10% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked and then incubated with primary antibodies against β-catenin and β-actin, followed by incubation with HRP-conjugated secondary antibodies. The membranes were developed in 3, 3'-diaminobenzidine. Images were captured using a gel imaging system and were analyzed using Quantity One.1, Control group; 2, 24 h after siRNA transfection; 3, 48 h after siRNA transfection; 4, 72 h after siRNA transfection; 5, 96 h after siRNA transfection.

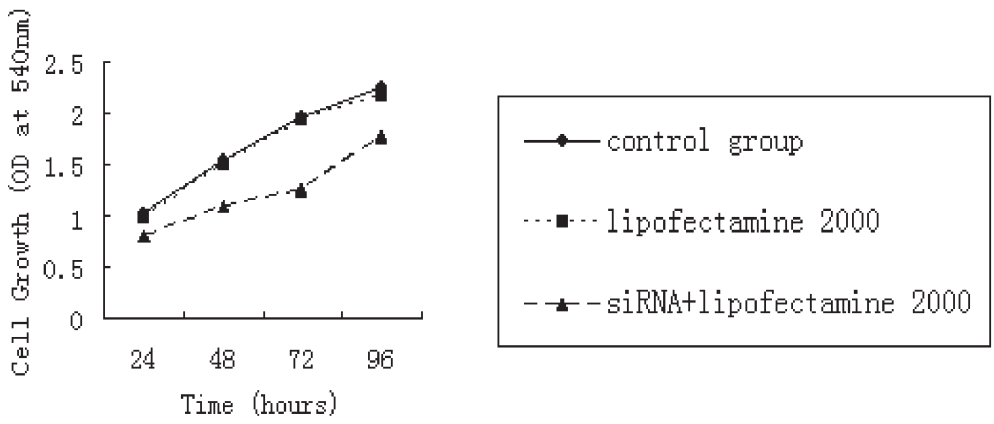

Figure 3. Numbers of viable cells at different time points. After 4 h incubation, medium was removed and DMSO was applied to each well and the sediments were redissolved. The absorbance was measured at 540 nm to determine the number of viable cells.

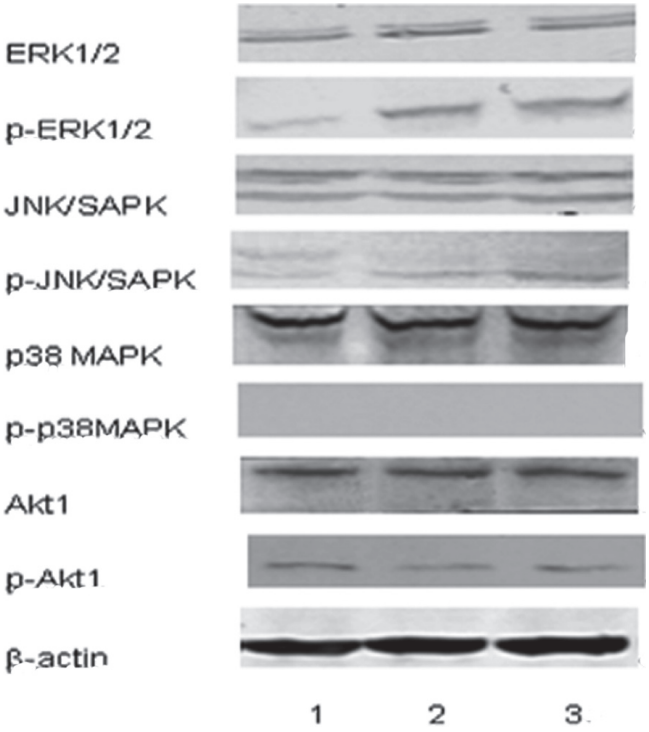

Figure 4. The protein levels of β-catenin, ERK1/2, p-ERK1/2, JNK/SAPK, p-JNK/SAPK, p38MAPK, p-p38MAPK, Akt1, p-Akt1 and β-actin were determined as described for Fig. 2. 1, Control group; 2, 24 h after siRNA transfection; 3, 48 h after siRNA transfection.

increased during the 24–72 h time period after siRNA transfection, while this effect was less at 96 h, suggesting recovery at this late time point (see Figure 3). The above findings suggested that short-term inhibition of cell growth or increased cell death has occurred as a result of transient transfection of siRNA.

MAPK and Akt protein expression. After siRNA transfection, MAPK and Akt protein levels was detected by Western blot at 72 and 96 h. ERK1/2 (p42/p44 MAPK) protein levels after transfection showed no significant difference compared to levels measured before transfection ($F = 4.37$, $P > 0.05$) In contrast, phosphorylated (p) ERK1/2 (p-p42 MAPK) protein levels increased slightly at 72 and 96 h after transfection ($t = 20.69$, $P < 0.05$). JNK/SAPK protein levels after transfection were the same as levels measured before transfection ($F = 1.73$, $P > 0.05$).. Levels of p-JNK/SAPK protein 54 kD form gradually decreased while levels of the 46 kD form gradually increased at 72 and 96 h after the transfection. p38 MAPK protein levels after transfection showed no significant difference compared to levels measured before transfection ($F = 0.05$, $P > 0.05$), and no p-p38 MAPK protein was observed in HepG2 cells. Akt1 protein levels showed no change following transfection ($F = 4.37$, $P > 0.05$), while p-Akt1 protein levels decreased at 72 h and increased again slightly at 96 h after transfection ($t = 12.25$, $P < 0.05$) (see Figure 4).

In short, after silencing of the β-catenin gene, ERK1/2, JNK/SAPK, and Akt1 protein levels showed no change, but their phosphorylated forms demonstrated significant changes. Thus, Wnt/β-catenin signaling pathway may regulate Ras/MAPK and PI3K/Akt signaling pathways by affecting the phosphorylation of ERK1/2, JNK/SAPK, and Akt1.

Discussion

To date, RNAi has been successfully applied to the study of gene function and has greatly aided in determining the interrelationships between upstream and downstream factors in different signaling pathways. RNAi may also be applied in future tumor therapies.

RNAi of certain genes often occurs within 24 h after transfection with the appropriate siRNA, and the level and duration of protein silencing depends on the turnover rate of the protein and half-life of the siRNA within the cells [22]. Harborth's studies indicated that the secondary and tertiary structures of the target mRNA also impacted siRNA silencing

efficiency [23]. Perhaps these attributes of siRNAs resulted in the gradual increase of β-catenin mRNA levels from 24 to 96 h, the gradual decrease of β-catenin protein levels from 24 to 72 h, and the slight increase of β-catenin protein levels at 96 h after transfection in our experiments.

Verma, et al. [24] designed an siRNA against β-catenin and transfected it into colon carcinoma cell lines SW_{480} and HCT_{116}. Their studies demonstrated that siRNA markedly decreased β-catenin gene expression and inhibited cell growth. However, in our experiments, the knockdown of β-catenin gene inhibited HCC cell growth at 24, 48, and 72 h, and this inhibition was decreased at 96 h, when the cell numbers began to increase again. Our results suggest that a one-time transient transfection may temporarily inhibit HCC cell growth or increase cell death, but as to whether or not multiple transfections or a stable transfection can cure HCC still requires further study. We speculate that these results may offer a new method and approach for HCC treatment. Because we observed that β-catenin protein levels were decreased, but the cell numbers still began to increase again at 96 h, we consider that other signaling pathways can likely compensate for this inhibition of Wnt/β-catenin pathway.

In our experiments, after blocking expression of the β-catenin gene, MAPK and Akt1 protein levels were not affected, while levels of their phosphorylated forms were affected. This observation indicated that Wnt/β-catenin signaling pathway participated in the regulation of Ras/MAPK and PI3K/Akt signaling pathway by affecting the phosphorylation state of ERK1/2, JNK/SAPK, and Akt1 proteins.

The MAPK family can be activated by stress, cytokines, G protein-coupled receptor activation, etc., and thereby plays an important role in a wide range of biological processes, including cell proliferation, apoptosis, angiogenesis, generation of cytokines, and other events. In our experiments, after introduction of siRNA directed against β-catenin into $HepG_2$ cells for 72 and 96 h, p-ERK1/2 protein expression increased gradually. The result indicated that silencing of the β-catenin gene activated p-p42 MAPK, resulting in a corresponding change in its target genes and promoting cell proliferation. This possibly compensated for the inhibited cell growth induced by the blockage of the β-catenin signaling pathway, resulting in restored cell division at 96 h. After the inhibition of β-catenin for 72 and 96 h, levels of the p-JNK/SAPK protein 54 kD form gradually decreased while the 46 kD form increased at 72 and 96 h post-transfection. These results showed that the β-catenin pathway affected JNK/SAPK phosphorylation, but the significance of this effect still needs to be studied further. Interestingly, there was no p-p38 MAPK protein detected in $HepG_2$ cells before or after transfection, indicating low p-p38 MAPK protein levels in $HepG_2$ cells that were not increased by silencing of β-catenin. The change in p-p38 MAPK expression needs to be validated in another HCC cell line.

A variety of growth factors, hormones, and cytokines, as well as phosphatase and tensin homolog (PTEN) inactivation and Ras activation, can stimulate Akt activation, further activating its downstream factors, such as bcl-2, P21, NF-κB, caspase-9, glycogen synthase kinase 3 (GSK3), etc. These downstream factors regulate cell growth, proliferation, differentiation, and metabolic pathways [25,26]. In our experiments, after silencing β-catenin, Akt1 protein levels showed no change, while p-Akt1 protein levels decreased at 72 h and increased again slightly at 96 h after transfection. Current studies indicate that the complex formed by PI3K, E-cadherin, β-catenin, and VEGFR-2 impacts Akt gene expression [27]. After β-catenin was blocked, the complex decreased, so p-Akt1activated from Akt1 was decreased at 72 h after transfection. With the time, some factors compensated for the β-catenin inhibitory effect resulting in p-Akt1 recovery at 96 h after transfection. These factors included XIAP [28] and Stat3 [29] that might activate Akt1 expression, thereby compensating for the p-Akt1 changes at 96 h after transfection. Changes in p-Akt1 levels may result in corresponding changes of downstream factors, thereby affecting tumor growth.

In summary, the Wnt/β-catenin signaling pathway may regulate cell growth and the protein expression of Ras/MAPK and PI3K/Akt signaling pathway components in the HCC $HepG_2$ cell line. These pathways compensate for the inhibitory effect of β-catenin, thereby affecting tumor cell growth and others downstream factors. The regulatory mechanism remains to be fully elucidated. These data will help us better understand HCC pathogenesis and the role of the Wnt/β-catenin signaling pathway during the pathogenesis of HCC. Furthermore, detailed studies about the process and relationship between these signaling pathways will increase our understanding of the pathogenesis, turnover, and prevention of tumors. By understanding these processes in detail, we may someday be able to treat tumors through modulation of the signal transduction cascades within the cells and specifically controlling cell growth, differentiation, and apoptosis.

Acknowledgment. Supported by: the International Joint Research Program of Science and Technology Department of Jilin Province (No. 20080724), China.

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