Aberrant expression and function of TCF4 in the proliferation of hepatocellular carcinoma cell line BEL-7402

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ABSTRACT

Wnt signaling pathway is essential for development and tumorigenesis, however, this signaling pathway in the progress of hepatocellular carcinoma (HCC) remains unclear. In this paper, we studied the function of human T-cell transcription factor-4 (TCF4), a key factor of Wnt signaling pathway, on the proliferation of HCC cell line. We showed that the expression of TCF4 mRNA in HCC cell line BEL-7402 was higher than that in immortalized normal liver cell line L02. Blockage of Wnt pathway by ΔNTCF4, a dominant negative TCF4, could suppress BEL-7402 cells growth and decrease the expression of *cyclin D1* and *c-myc*, two of target genes of Wnt pathway. On the other hand, stimulating Wnt pathway by introducing a degradation-resistant β-catenin S37A could increase BEL-7402 cells proliferation. But all the treatments had no effect on L02 cells. Our data indicated that TCF4 might be another key factor in Wnt pathway involved in HCC cells proliferation and TCF4 could be an effective therapeutic target for suppressing the growth of hepatocellular cancers.

Keywords: Wnt signaling pathway, β-catenin, dominant negative TCF4.

INTRODUCTION

Wnt signaling pathway plays a critical role in directing cell fates during embryogenesis. In addition, inappropriate activation of Wnt signaling pathway results in a variety of human cancers by turning on genes encoding oncoproteins and cell-cycle regulators[1-3]. The protein β-catenin is a major component of the Wnt signaling pathway, which is involved in two major functions: cell adhesion and Wnt signal transduction[4, 5]. In the absence of Wnt signal, β-catenin is localized in cell-cell junctions but very low in cytoplasm and nucleus because excess β-catenin in cytoplasm is targeted to proteasome-mediated degradation by a complex containing GSK-3 β[6], Axin[7] and APC[8]. Activation of Wnt signaling results in the inactivation of GSK-3 β, which in turn leads to the stabilization of cytoplasmic β-catenin. The accumulation of β-catenin promotes its nuclear translocation where it associates with LEF/TCF transcription factors and activates Wnt target genes[9, 10].

HCC is one of the most common fatal cancers worldwide. Inappropriate activation of Wnt signaling pathway has been implicated recently in the development of HCC. β-catenin gene mutation was often found in HCC, which

resulted in the protein accumulation in cytoplama and thus activating of Wnt signaling pathway[11]. Several components related to β-catenin degradation were also found to be mutated during hepatocellular carcinogenesis. Mutation in *Axin* gene resulting in β-catenin accumulation has been reported in HCC[12]. But APC gene was rarely mutated in HCC although its mutation occurred in most colon cancers[12].

So other component of Wnt signaling pathway might be involved the proliferation of HCC cell. The formation of a complex between β-catenin and transcription factors of TCF/LEF family is a key regulatory event in the Wnt signaling pathway[13]. TCF/LEF family include LEF-1, TCF1, TCF3, TCF3 a, TCF4, which all contain DNA-binding high mobility group (HMG) domain and a β-catenin binding domain[14]. TCF4 is an important member of this family. The N-terminal 53 amino acids of TCF4 bind with high affinity to β-catenin[15]. TCF4 and β-catenin form a complex to transactivate target genes expression including cell cycle regulating genes cyclin D1 and c-myc. We found that Wnt signaling was aberrantly activated in HCC cells BEL-7402 because both the high expression of TCF4 and the accumulation of β-catenin were observed. The results suggested that activation of TCF4 coupled with β-catenin expression may contribute to the development ofHCC. It was also worth noting that TCF4 may be a potential target of gene therapy for HCC.

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MATERIALS AND METHODS

Cells, plasmids and antibodies

Immortalized normal human liver cells L02 and HCC cells BEL-7402 were purchased from Shanghai Institute of Cell Biology. They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% NCS. The cultures were kept in a 5% CO_2 and 95% air humidified incubator at 37°C.

The wild type TCF reporter plasmid (TOPFLASH) and mutated reporter plasmid (FOPFLASH) were obtained from Upstate biotechnology (NY, USA). The cDNA encoding β-cateninS37A was a gift of Dr. Xin Yuan FU and subcloned into expression vector pcDNA3. The control reporter plasmid β-gal-CMV was a gift of Dr. Zhi-Ming YIN . The constructs of pcDNA3TCF4, pcDNA3 dominant negative TCF4 and the CyclinD1 reporter plasmid were constructed by Dr. O. Tetsu and Dr. F. McCormick who got the human TCF4 cDNA fragment and cyclinD1 promoter fragment from Dr. H. Clevers, Dr. A. Arnold and Dr. R. Pestell.

Anti-β-catenin polyclonal antibody, anti-cyclinD1 monoclonal antibody and anti-c-Myc monoclonal antibody were obtained from Santa Cruz (California, USA). Anti-β-tubulin antibody was purchased from Sigma (MO, USA).

Immunofluorescence staining of hepatoma cell lines

The HCC cell line BEL-7402 and the normal liver cell line L02 were washed in PBS, then fixed and permeabilized. Cells were incubated for 1 h in blocking solution [PBS, 3% BSA (wt/vol)], and then with a rabbit polyclonal anti- β-catenin antibody (dilution 1:200) at room temperature in a moist chamber. The cells were washed several times in PBS-0.05% (vol/vol) Tween 20, and incubated for 45 min with anti-rabbit fluorescein isothiocyanate antibody (dilution 1:500). The cells were examined under a leica fluorescence microscope and photographed. For quantitation of β-catenin in cytosole, the fluorescence intensity was measured with Image Analysis Software. At least 50 cells were examined in each experiment.

Western blot analysis

Cells were washed and lysed in lysis buffer (20 m*M* Tris-HCl, pH 8.0, 75 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1.0% NP-40, 1 m*M* PMSF, 10 μg/ml leupeptin and10 mg/ml aprotinin). Protein concentrations were determined by Bradford assays. Samples of extract containing equal amounts of total protein were resolved on 8.0% (wt/vol) SDS-PAGE and transferred to nitrocellulose membrane, which was blocked with 3% blocking buffer and then was incubated with polyclonal anti-β-catenin (dilution 1:500), monoclonal anti-cyclinD1 (dilution 1:500) and monoclonal anti-c-Myc (1:500) respectively. Then the membrane was incubated with the peroxidase-conjugated secondary antibody for 40 min. The blotting patterns were revealed using the ECL system (Roche, Mannhein, Germany). β-tublin was blotted as a control of protein loading.

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from cells using Tripure reagent (Roche, IN, USA). The total RNA (1 μg) was used for a reverse transcriptional reaction (Promega, WI, USA). The primers sequence used for amplification of LEF-1 and TCF-4 were: LEF-1 F, 5'-CCAGCTA-

TTGTAACACCTCA-3', LEF-1R, 5'-TTCAGATGTAGGCAG-CTGTC-3'; TCF-4F, 5'-TTCAAAGACGACGGCGAACAG-3', TCF-4R, 5'-TTGCTGTACGTGATAAGAGGCG-3'. Cyclophilin was amplified as internal control using following primers: F, 5'-GACAGCAGAAAACTTTCGTGC -3', R, 5'- TCCAGCCA-CTCAGTCTTGG-3'. The amplification of the LEF-1, TCF4 and Cyclophilin cDNAs was performed using Taq polymerase (Promega, WI, USA) with the following thermocycle parameters: 5 min at 94°C and 25cycles of 40s at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by a final incubation at 72°C. RT-PCR amplified fragments (LEF-1, 453bp; TCF4, 517bp, Cyclophilin, 600 bp) were analyzed by 2% agarose gel electrophoresis. Densitometric scanning of the bands was carried out by Syngene Gel image scanning system (Syngene, England). The resulting bands were quantified, and the relative amount of LEF-1 and TCF4 mRNA was calculated after normalization with cyclophilin mRNA detected in the same sample.

Transfection, luciferase assay

Transient transfection was carried out by the calcium phosphate precipitation method. For luciferase assays, β-galactosidase expression plasmid was cotransfected as an internal control to normalize transfection efficiency. Luciferase activities in cell lysates were determined with luminometer (Lumat LB 9507, Berthold, Germany) 48 h after transfection using a luciferase assay kit (Promega, WI, USA). Each transfection was performed in triplicate and was repeated at least three times.

Colorimetric cytotoxicity assay

Analysis of cell proliferation was performed by SRB assay. Cells (1×10^4 cells per well) were seeded into 24-well plates 24 h before β-catenin or TCF4 plasmids transfection. The SRB proliferation activity assay was performed 48 h after transfection as described by Skehan[16] with slight modifications. Cultured cells fixed with trichloroacetic acid were stained for 30 min with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 m*M* unbuffered Tris base (pH10.5) for 5 min. OD value was read at 490nm in Elx800 universal microplate reader (Bio-tech instrument, VT, USA).

Colony formation assay

BEL-7402 cells and L02 cells were transfected with dominant negative TCF-4 and β-cateninS37A, respectively. After 14 days selection with G418, cells were stained with 0.5% crystal violet in 20% ethanol and photographed. The morphology and the number of colonies were counted under stereomicroscope.

Statistics

Data were presented as mean ± standard deviation (SD). Statistical differences between the two groups were evaluated using the paired Student's t test. P values less than 0.05 were considered significant difference.

RESULTS

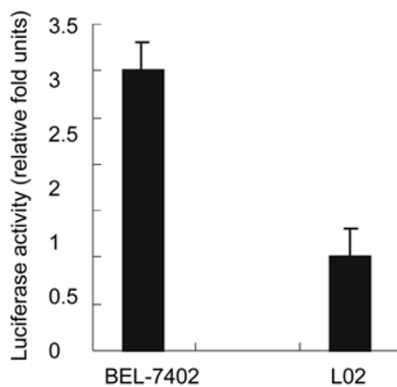### Wnt signaling pathway was activated in HCC cell line BEL-7402

Fig 1. The Wnt pathway was activated in BEL-7402 cells determined by TOPFLASH reporter plasmid. BEL-7402 cells and L02 cells were transfected with the reporter plasmid (TOPFLASH or FOPFLASH). β-gal reporter construct was co-transfected to each sample to normalize transfection efficiency. The fold of BEL-7402 cells reporter activity to L02 cells reporter activity was indicated.
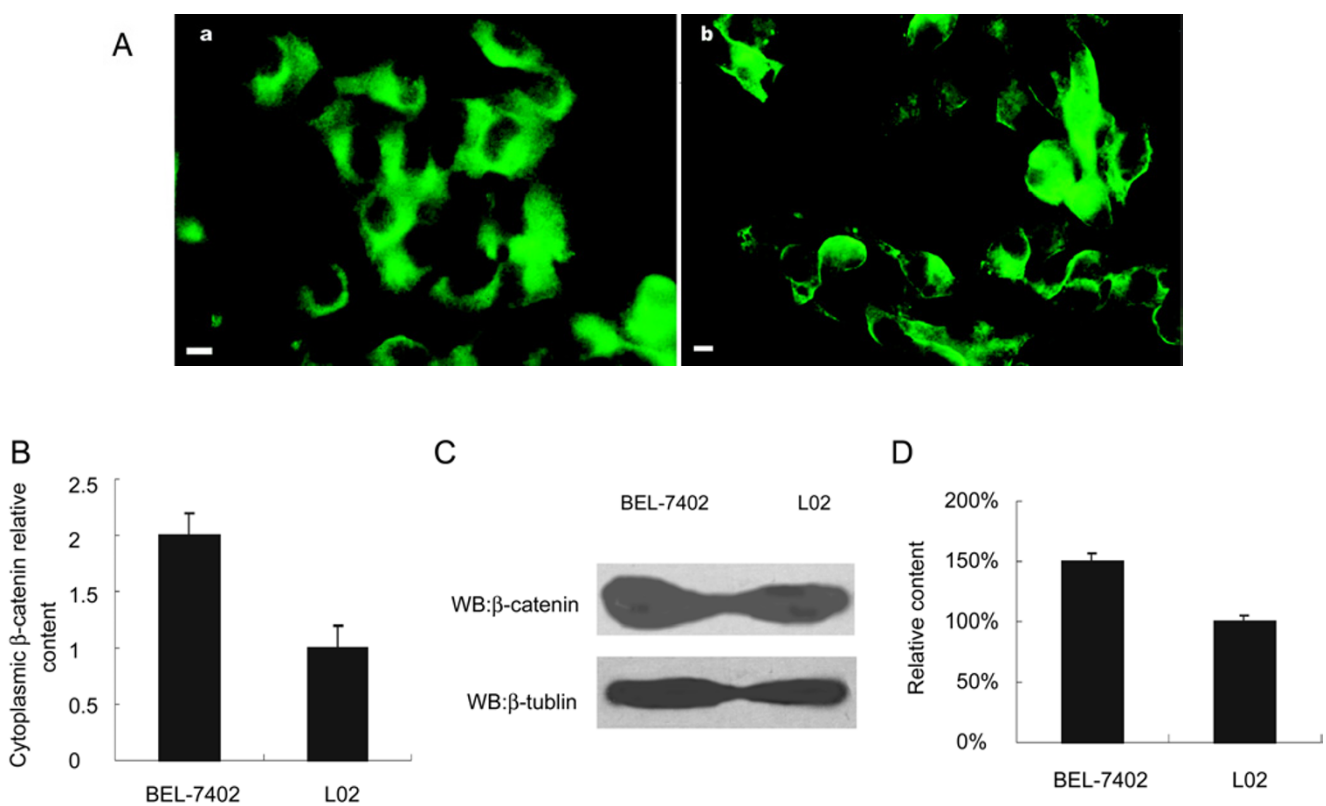

Fig 2. Expression of β-catenin protein in BEL-7402 cells detected by Western blotting analysis and Immunnofluorescence assay (**A**) Immunnofluorescence assay with β-catenin antibody in BEL-7402 cells and L02 cells. (**a**) BEL-7402 cells; (**b**) L02 cells. Bar, 10 μm (**B**): Quantitative analysis of the immunofluorescence image in cytoplasmic β-catenin level of BEL-7402 and L02 cells. (**C**) BEL-7402 cells and L02 cells were lysed by cell lysis buffer and the isolated protein was subjected to immunoblotting with β-catenin antibody (upper panel) and β-tublin antibody as loading control (lower panel). (**D**) Expression levels relative to control β-tublin level were analyzed according to quantitative analysis of data of Fig 2C.

L02 and BEL-7402 are extensively used HCC cell line in hepatoma research area in China, but the genetic background and the situation of Wnt components remain largely unknown. So we first investigated the activation of Wnt pathway by TOPFLASH luciferase reporter assay. As shown in Fig 1, the transcriptional activity of the TOPFLASH reporter in BEL-7402 was about 3 times higher than that in L02 cells, and no enhanced transcription was detected in cells transfected with the negative control FOPFLASH (not shown). The results suggested that Wnt
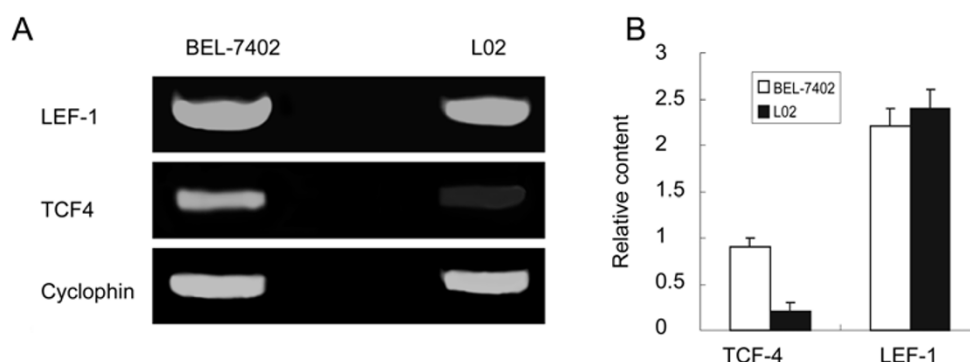
Fig 3. Predominant expression of TCF4 mRNA in BEL-7402 cells by RT-PCR analysis (**A**) One microgram of total RNA extracted from BEL-7402 or L02 cells was used for reverse transcription. PCR was then performed with the primers as described in Meterials and Methods. (**B**) Expression levels relative to control cyclophin mRNA level were analyzed according to quantitation of data of Fig 3A.
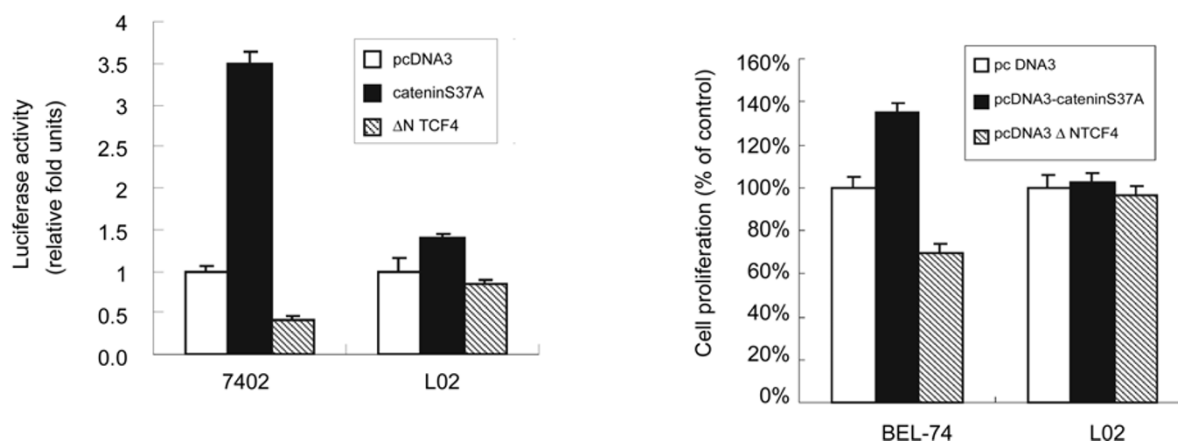

Fig 4. Effect of cateninS37A and ΔNTCF4 on Wnt pathway in BEL-7402 cells and L02 cells Reporter gene assay with TOPFLASH in BEL-7402 cells and L02 cells. Cells were transfected with 0.5μg TOPFLASH, co-transfected in the combination of 1 μg β-cateninS37A or βΔNTCF4 constructs. β-gal reporter construct was co-transfected to normalize the transfection efficiency.

Fig 5. Effect of β-catenin and ΔNTCF4 on the growth of BEL-7402 and L02 cells BEL-7402 cells and L02 cells were transfected with β-catenin or ΔNTCF4 construct. The proliferation of the cells was determined by SRB assay.

pathway was activated in HCC cell lines.

The accumulation of β-catenin and high expression of TCF4 mRNA in HCC BEL-7402 cells

To define which component of Wnt signaling pathway was changed in HCC cell line BEL-7402, we first used immunofluorescence staining to test the subcellular localization of β-catenin. Fig 2A showed that BEL-7402 cells were intensively stained in the cytoplasm, but not in nucleus, with very weak membrane staining comparing with L02 cells. The fluorescence intensity of β-catenin in BEL-7402 cytosole was 2 fold higher than that in L02 cells (Fig 2B). This results was confirmed by western blotting of β-catenin shown in Fig 2C and 2D that β-catenin level was 50% higher in HCC cells BEL-7402 than that in L02 cells (p<0.05). This suggested that the inap-

propriate activation of Wnt pathway in BEL-7402 cell line was at least partially due to the accumulation of β-catenin.

The accumulated β-catenin need to bind to LEF/TCF transcription factors to activate the target gene expression, so we further examined whether LEF/TCF transcription factor was also involved in the activation of Wnt pathway in HCC cells. We checked the mRNA level of LEF1 and TCF4 in BEL-7402 cells and L02 cells by semi-quantitive RT-PCR analysis. Fig 3 showed that TCF-4 mRNA was 3 times higher expressed in BEL-7402 cells than that in L02 cells, in contrast, LEF-1 mRNA level in BEL-7402 cells did not change comparing with that in L02 cells. The result suggested that the aberrant Wnt pathway may be due to both the enhanced expression of TCF4 gene and the accumulation of β-catenin.
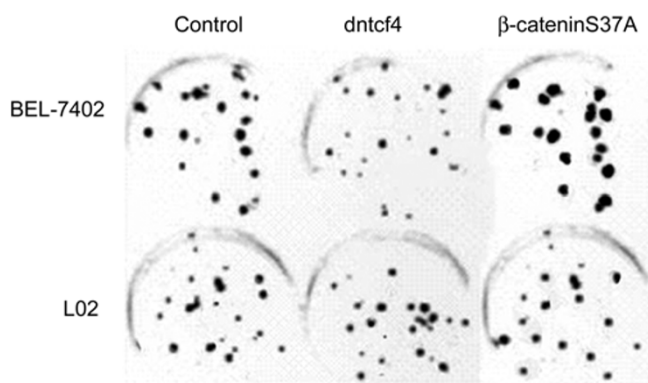
Fig 6. Colony-formation assay on BEL-7402 cells and L02 cells transfected with dominant-negative TCF-4 and cateninS37A BEL-7402 cells and L02 cells were transfected with dominant-negative TCF-4 and cateninS37A and selected with G418, respectively. After 14 days's selection in G418, cells were stained and photographed.

The accumulation of β-catenin stimulated BEL-7402 cells proliferation

To further evaluate the function of accumulated β-catenin on BEL-7402 cell proliferation, we constructed the β-cateninS37A mutant plasmid, whose serine residue (S37) was mutated into alanine residue. Thus the phosphorylation of β-catenin by GSK-3 β is inhibited and then the subsequent ubiquitination is blocked, which leads to β-catenin accumulation. As shown in Fig 4, the reporter gene assay showed that the Wnt pathway was highly activated by β-cateninS37A overexpression in BEL-7402 cells, but only slightly activated in L02 cells. The SRB assay (Fig 5) also showed that the proliferation of BEL-7402 cells was accelerated by β-cateninS37A overexpression but that of L02 cells was only affected slightly. The result was further confirmed by the colony-forming assay (Fig 6). BEL-7402 cells expressing β-cateninS37A resulted in the increase of colony size, but no change in the number of colonies. But L02 cells expressing β-cateninS37A had no change in the number and size of colonies. These results suggested that β-catenin played important role in BEL-7402 cells proliferation.

β-catenin governed BEL-7402 cells proliferation by regulating cyclinD1 protein level and c-Myc protein level

We further postulated that β-catenin promoted cell proliferation by activating *cyclin D1* and *c-myc*, two of target genes of Wnt pathway. The luciferase reporter assay (Fig 7A) and western blotting analysis (Fig 7 B) showed that the expression of cyclin D1 and c-Myc were increased by β-cateninS37A in BEL-7402 cells, but not in L02 cells.
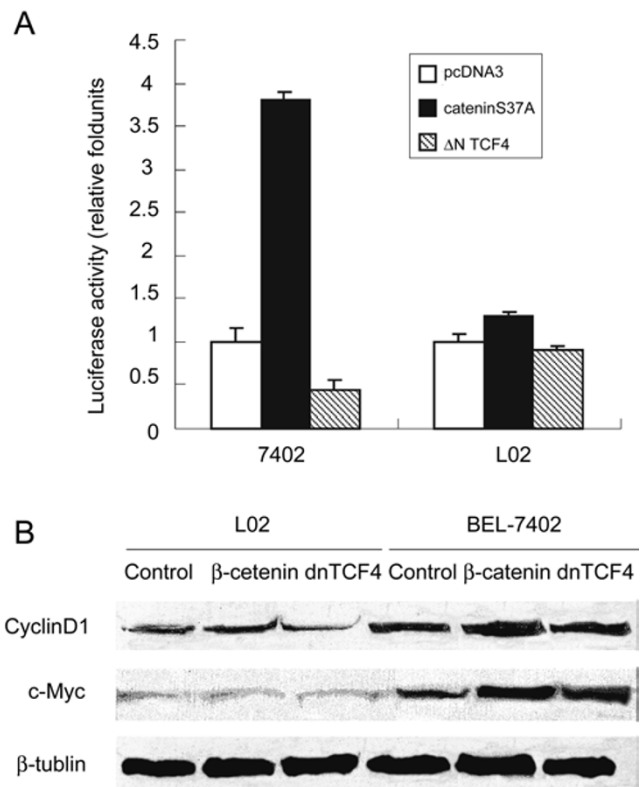

Fig 7. β-cateninS37A and DN TCF4 affect *cyclin D1* and *c-myc* expression (**A**) Cyclin D1 reporter construct was introduced into BEL-7402 cells and L02 cells together with β-cateninS37A or ΔNTCF4. β-gal construct was co-transfected as control. The assay was performed 36 h after transfection. (**B**) Western blotting analysis of cells transfected with β-cateninS37A and dnTCF4. Cell extracts were prepared 48 h after transfection and the blot was first probed with cyclin D1 antibody and c-myc antibody. Subsequently, the identical membrane was probed with β-tublin antibody to indicate that equal amounts of protein loaded in each lane.

The result indicated that high expression of cyclinD1 and c-Myc caused by Wnt pathway activation was the reason of BEL-7402 cell proliferation.

Dominant negative TCF4 mutant suppressed BEL-7402 cells proliferation and decreased cyclinD1 and c-Myc expression

To test whether TCF 4 was necessary to BEL-7402 cells growth, loss of function studies were carried out with a dominant negative TCF4 (DNTCF4). The TCF4 mutant deleted 2-53 amino-terminus residues which was β-catenin binding domain. This is a dominant-negative TCF4 form that can block Wnt signal pathway (Fig 4). DNTCF4 also could inhibit the proliferation of BEL-7402 cells (Fig 5) and decrease the colonies' size of BEL-7402 cells (Fig 6). As shown in Fig 7, DNTCF4 overexpression could lead to an decreased expression of the cyclinD1 and

c-Myc in BEL-7402 cells. The results suggested that dominant negative TCF4 could suppress HCC cells proliferation and TCF4 could be used as a gene therapy target of HCC.

DISCUSSIONS

The aberrant activation of the Wnt-β-catenin pathway was noteworthy in HCC carcinogensis[18, 19]. β-catenin gene mutation and protein accumulation in HCC has been reported[19], and it has been shown that the accumulation of β-catenin may be the results of the mutation of either *APC, β-catenin* or *AXIN1* gene in Wnt pathway [11]. In this report, we confirmed that Wnt pathway was really inappropriately activated in BEL-7402 cells and this activation accounted for the accelerated proliferation of BEL-7402 cells. But activation of Wnt pathway by overexpre-ssion β-cateninS37A had no effect on L02 cells, so there might be other components of Wnt pathway governing the proliferation of BEL-7402 cells. Harada et al [21] also showed that the Wnt pathway activation by stabilizing β-catenin was not sufficient for hepato-carcinogenesis, additional mutations or epigenetic changes may be required.

TCF4, an important molecule of Wnt pathway, is a member of TCF family and plays many important roles in tumorigenesis[20]. TCF-4 encodes a transcription factor that interacts functionally with β-catenin to transactivate target genes expression. High level of TCF-4 expression has been identified in colon cancer, mammary carcinoma and a variety of colorectal cancer cells[22]. In the present study, we have found that TCF4 mRNA was predominant expressed in BEL-7402 cells. Further studies demonstrated that the transcriptional activity of Wnt pathway could be blocked by ΔNTCF4 overexpression. This indicated that TCF4 might be another key factor in Wnt pathway involved in HCC cells proliferation. This point was confirmed by further experiments that both the proliferation and colony formation assay of HCC cells were suppressed by ΔNTCF4 overexpression.

But we still can't exclude the possibility that other components of Wnt signal pathway altered in BEL-7402. It has been reported that there are β-catenin, and *AXIN1* gene mutation found in HCC[12]. But we know nothing about the genetics background of BEL-7402 cell. The aberrant expression besides the gene mutation of the components of Wnt signal pathway might also account for the control of BEL-7402 cell proliferation.

Cyclin D1 and *c-myc* are two cell cycle progression genes. *Cyclin D1* contains a TCF response element within its promoter region and is thought to be a rate-limiting mediator of the G1 to S phase transition in the cell cycle and be implicated in the development of cancer[23]. C-myc, a downstream target of the Wnt pathway, contributes to progression of a wide range of human and animal tumors [24]. C-Myc executes its multiple activities mostly through transcriptional regulation of the target genes. We showed that expression of cyclin D1 and c-Myc were strongly dependent on β-catenin expression in BEL-7402 cells. Blockage of Wnt pathway by ΔNTCF4 would result in growth arrest and expression decrease of cyclin D1 as well as c-Myc in BEL-7402 cells. These can explain why the proliferation of BEL-7402 cells could be stimulated by the accumulation of β-catenin and blocked by ΔNTCF4.

If these observations can be confirmed in other HCC cell lines, we suggested that TCF4 may be an effective therapeutic molecule for suppressing growth of HCC. Disruption of the TCF4-β-catenin interaction could be useful in treating HCC, and this would provide a new insight into the gene therapy of HCC.

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