Dickkopf-1 contributes to the tumorigenesis of hepatocellular carcinoma by activating the Wnt/β-catenin signaling pathway

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The supplementary data

1. Supplementary Information Figure

2. Supplementary Materials and Methods

3. Supplementary Table 1–4
Supplementary Fig. 1 Results regarding soluble DKK1 levels and the DKK1 deletion downregulation of TCF/LEF activity. Histological features and the expression of DKK1 in subcutaneous tumors.

The level of DKK1 was increased in the supernatant of cultured stable forced expression DKK1 HepG2(a) and HUH-7 cells (b) and vice versa. TCF/LEF-driven transcription activity was measured as an indicator of Wnt/β-catenin signaling in HepG2 and HUH-7 cells in vitro. Suppression of DKK1 in HepG2 (c) and HUH-7 (d) cells decreased the luciferase activity and vice versa. LiCl served as a positive control (10 mM). Histological features of the tumors indicate poor HCC
differentiation (e). The expression of DKK1 was much higher in subcutaneous tumors (f). *P<0.05, **P<0.01

Supplementary Fig. 2 Western blotting results quantified by ImageJ.

The protein levels of DKK1 in DKK1-shRNA and LV-DKK1 HCC cells were quantified by ImageJ (a, b). The protein levels of Wnt1 and β-catenin in DKK1-shRNA and LV-DKK1 HCC cells were quantified by ImageJ(c, d). The protein levels of nuclear β-catenin in DKK1-shRNA and LV-DKK1 HCC cells were
quantified by ImageJ (e). The protein levels of β-catenin in β-catenin-shRNA HCC cells were quantified by ImageJ (f). *P<0.05

2. Supplementary Materials and Methods

Ethics approval and consent to participate

Informed consent was obtained from all subjects according to the Internal Review and Ethics Boards of Sun Yat-Sen Memorial Hospital, and the project was in accordance with the Helsinki Declaration of 1964. All animal experimentation described in this study was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University.

Patients and immunohistochemistry

Samples of cirrhotic and tumor tissues were used in tissue microarray assays (TMAs). These samples were obtained from twenty-two cirrhotic patients and fifty-three consecutive HCC patients who underwent resection at the Department of Hepato-Pancreato-Biliary Surgery, Sun Yat-sen Memorial Hospital. Thirty-three pairs of fresh tumor tissues and the corresponding peritumoral tissues were randomly chosen from the tissue bank of Sun Yat-sen Memorial Hospital and were used for qRT-PCR analysis. None of the patients received chemotherapy or radiation therapy prior to the radical tumor resection. The differences and significance of DKK1 expression in these patients were investigated. The clinical and histopathological data, including gender, age, functional state of the liver, size of the tumor and tumor
number, are presented in Table 1. The tumor stage and histological grade were classified according to the criteria defined by the American Joint Committee on Cancer (AJCC) 7th Edition (2010). Routine histological examinations were performed with hematoxylin and eosin staining (H&E).

TMAs were constructed and immunohistochemistry was performed as described\textsuperscript{25}. Details regarding primary and secondary antibodies are provided in Supplementary Table 1. DKK1 and β-catenin expression was evaluated under a light microscope at a magnification of $200 \times$. For each specimen within the TMAs, five images of representative areas were acquired and tumor cells were counted. The percentages of positively stained cells were determined by examination under a microscope of 30 randomly selected foci, which were each composed of more than 100 cells. The intensity of DKK1 staining was evaluated as follows: strongly positive samples (scored as ++++) had dark brown staining in > 50% of cells, completely obscuring the cytoplasm; moderately positive (scored as ++) had dark brown staining in 25% to 50% of cells obscuring the cytoplasm; weakly positive (+) showed a lesser degree of brown staining in the tumor cell cytoplasm; and “absent” (scored as -) exhibited no appreciable staining in cells. Strong and moderate scores were regarded as positive results.\textsuperscript{15, 20}

Cell lines and cell culture

Two human HCC cell lines HepG2 and HUH-7 cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Invitrogen Co., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine
serum (Invitrogen) as recommended by the supplier. All cultures were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

Quantitative RT-PCR and Western Blot analysis

Total RNA was isolated using RNAiso Plus reagent according to the manufacturer’s protocol (TaKaRa, Tokyo, Japan). Primer sets used for PCR amplification were shown in supplement Table 2. As a control, the levels of glyceraldehyde phosphate dehydrogenase (GAPDH) expression were also analyzed. Real-time PCR was performed as described.²⁵

Total protein extraction and western blot analysis was also performed as described in our previous study.²⁵ Briefly, Protein lysates obtained from the cultured cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were probed with primary antibodies recognizing DKK1 (1:2000), WNT1 (1:2000), β-catenin (1:5000) and β-Tubulin (1: 1000). After incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch, USA), protein bands were visualized using enhanced chemiluminescence (ECL) plus Western blotting detection reagents followed by exposure to Hyper-films (Amersham, UK). Details regarding the primary and secondary antibodies are provided in Supplementary Table 1.

Nuclear protein extraction was performed as described in the Supplementary Materials and Methods. The results of western blot were quantified by Image J (Supplementary Fig. 3).
**Nuclear protein extraction**

Nuclear protein extraction was performed as follows: Wash plates with ice-cold PBS twice. Aspirate off all PBS. Tilt plates on ice for an additional 1 min to remove all remnants of PBS. Lyse cells in an appropriate volume (10x cell volume) of ice-cold buffer A containing 0.5% NP-40, 1mM DTT and 1x protease inhibitor (PI). Harvest cell lysates with a cell scraper. Transfer this lysate to a pre-chilled 1.5ml eppendorf tubes on ice. Pipet up and down several times to disrupt cell clumps. Rotate 10min at 4℃. Centrifuge at top speed for 3min at 4℃. Save supernatant (cytosolic fraction) if desired. Otherwise discard the supernatant. Add 500ul ice-cold buffer A, votex 10 seconds. Spin at top speed for 3min at 4℃. Estimate the volume of nuclear pellet and add 3x volumes of ice-cold buffer B containing 1mM DTT and 1x PI. Incubate on ice of 30 min with intermittent strong vortexing. Spin for 15min at top speed at 4℃. collect supernatant and measure the protein concentration with BCA assay. Aliquot the extract and keep in -80℃ for future use.

**Cell proliferation assay and colony Formation Assay**

Cells (5 × 10³ /well) were cultured in 96-well tissue culture plates until they reached 50% confluence. Cell viability was determined using a Cell Counting Kit-8 (CCK-8) purchased from Dojindo Molecular Technologies (Gaithersburg, MA). Briefly, 10 μl of water-soluble formazan dye was added to each well and incubated for 2 h. The absorbance at 450 nm was measured by an enzyme linked immunosorbent assay
(ELISA) plate reader. The absorbance of the negative control (OD) was considered to be 0.

HCC cells were plated in triplicate in six-well plates. After 7 days, the cells were rinsed with PBS twice, fixed with 10% formaldehyde, and stained with 0.1% crystal violet in 10% ethanol and the numbers of colonies were counted.

**Cell cycle analysis**

The cell cycle was analyzed using flow cytometry with propidium iodide (PI; Sigma, USA) staining. For each group, the cells were harvested and washed with phosphate-buffered saline (PBS) and fixed overnight in ice-cold 70% ethanol. The cells were washed twice with PBS and treated with 1 mg/L RNaseA (TaKaRa, Japan) for 15 min. Finally, the cells were stained with 50 mg/L PI in the dark for 1 h. Then, the cell cycle analysis was performed using a fluorescence-activated cell sorter (BD Biosciences, Franklin Lakes, NJ, USA), and the PI fluorescence was measured at 488 nm. Each group was analyzed in triplicate, and at least 10,000 cells were analyzed in each experiment.

**Cell invasion assay**

The invasive activity of HepG2 and SMMC-7721 cells was estimated using transwell plates (6.5 mm in diameter, polycarbonate membrane, 8 μm pore size) coated with extracellular matrix gel obtained from Corning (Corning, NY, USA). Twenty-four hours after transfection, an aliquot of $1 \times 10^5$ cells was placed in the upper chamber with 0.1 ml of serum-free medium, whereas the lower chamber (of a 24-well plate)
was loaded with 0.5 ml of medium containing 10% fetal bovine serum. After 24 h of incubation, the cells were fixed with 4% paraformaldehyde and then counterstained with 0.1% crystal violet. The cells that had migrated into the lower chambers were observed and counted under a light microscope. Then, the number of migratory cells was calculated.

**In vivo subcutaneous xenografts assay**

Briefly, $5 \times 10^6$ cells were suspended in 100 µl PBS and were injected subcutaneously into 4-weeks old female nude mice (Balb/c nu/nu). Tumor volumes were monitored every 7 days by measuring the length and width with a caliper and using the formula $(\text{width}^2) \times \text{length}/2$. Mice were sacrificed 8 weeks after injection, and the tumors were isolated and measured. All animals in our laboratory received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences and published by the National Institutes of Healthy.
### 3. Supplementary Table 1: The information of antibodies used in this study

| Antibodies | Company     | Product Number | Dilution              |
|------------|-------------|----------------|-----------------------|
| DKK1       | Abcam       | ab109416       | 1:300 (IHC) 1:2000 (WB) |
| β-catenin  | Abcam       | ab32572        | 1:500 (IC) 1:5000(WB)  |
| β-tubulin  | Abcam       | ab179513       | 1:1000(WB)            |
| Wnt1       | Abcam       | ab91191        | 1:2000(WB)            |
| Histone H3 | Abcam       | Ab4076         | 1:1000(WB)            |
| HRP-linked anti-mouse IgG | Cell Signaling Technology | #7076 | 1:2000(WB) |
| HRP-linked anti-rabbit IgG | Cell Signaling Technology | #7074 | 1:2000(WB) |
**Supplementary Table 2: Primers used in the qPCR.**

| Genename | Type   | Sequence 5'-3'                      |
|----------|--------|-------------------------------------|
| DKK-1    | Forward| GACCCAGGCTTGCAAAAGTGAC              |
|          | Reverse| GCCCAGAGCCATCATCTCAG                 |
| GAPDH    | Forward| ACAACTTTGGTATCGTGGAAG                |
|          | Reverse| ACAACTTTGGTATCGTGGAAG                |
### Supplementary Table 3: Oligonucleotides and primer sequences for generation of Lentiviruses

| Name         | Type  | Sequence (5’-3’)                                      |
|--------------|-------|------------------------------------------------------|
| DKK1-shRNA   | Forward | GATCCGTACCAGCATAGGAGAAAATTCAGAGAT                   |
|              |        | CATCATTCAAGGCAGTATTACTTTTTTG                        |
|              | Reverse| AATTCAAAAAAAGTAATACTGCCTGAATGATGAT                  |
|              |        | CTCTTGAATCATCATTACCAGGCGAGTATTACG                   |
| β-catenin-shRNA | Forward | GATCCGTCTAACCTCATTGCAATAATTTCAAG                   |
|              |        | AGAACGTGACACGTTCCGAGAACCTTTTTT                      |
|              | Reverse| AATTCAAAAAAAGTTCTCCGAACGTGTCACGTTTC                |
|              |        | TCTTGAACGTGACACGTTCCGAGAACG                        |
| Scramble     | Forward | GATCCGTTCCTCCGAACGTGTCACGTTTCAAGAG                 |
|              |        | AACGTGACACGTTCCGAGAACCTTTTTT                      |
|              | Reverse| AATTCAAAAAAAGTTCTCCGAACGTGTCACGTTTC                |
|              |        | TCTTGAACGTGACACGTTCCGAGAACCG                      |

The underlined parts of primers indicate the specific cleavage sites of restriction enzymes ("", EcoRI; “”, BamHI).

NCBI Reference Sequence of DKK1: NM_012242.2
## Supplementary Table 4: Primer sequences for amplification of TCF/LEF

| Gene name | Type | Sequence (5'-3') |
|-----------|------|-----------------|
| TCF/LEF   | *MluI* Forward | cgcgtCCTTTGATCTTTACCCCCTTTGATCTTACCCCTTTTGGATCTTACCCCTTTGATCT |
|           | *XhoI* Reverse  | TACCc |
|           | *XhoI* Reverse  | tcgagGGTAAGATCAAGGGGGTAAGATCAAGGGGGTAAGAT |
|           |                 | CAAAGGa |