Wnt Antagonist Gene *DKK2* Is Epigenetically Silenced and Inhibits Renal Cancer Progression through Apoptotic and Cell Cycle Pathways

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Abstract

Purpose: Wnt/β-catenin signaling is involved in renal cancer. DKK2, a Wnt antagonist, is silenced in some cancers, although its function has not been investigated. We hypothesized that DKK2 may be epigenetically silenced and inhibits progression of renal cell carcinoma (RCC).

Experimental Design: RCC cell lines and a normal kidney cell line were used for methylation and chromatin immunoprecipitation assays. To assess various functions of DKK2, we established stable *DKK2*-transfected cells and examined them with regard to cell viability, colony formation, apoptosis, cell cycle, and invasive capability. A total of 52 patients with confirmed conventional RCC were enrolled in this study.

Results: RCC cell lines had decreased levels of DKK2, which were significantly increased after treatment with 5-Aza-2′-deoxycytidine alone or 5-Aza-2′-deoxycytidine and trichostatin A. In chromatin immunoprecipitation assay, the levels of acetyl H3, acetyl H4, and dimethylated H3K4 were decreased, whereas the level of dimethylated H3K9 was increased in RCC cell lines compared with HK2 cells. Increased methylation in RCC tissues was associated with higher grades, pathologic stages, and pathologic tumor in RCC. Functional analysis showed that the numbers of viable A498 cells were significantly decreased in *DKK2*-transfected cells compared with mock cells. The number of apoptotic cells and S/G_2-M phase cells was significantly increased and decreased after *DKK2* transfection, respectively. Corresponding to these results, Bcl2 and cyclin D1 expression were also decreased in DKK2-overexpressing cells.

Conclusion: DKK2 is epigenetically silenced by methylation in higher grades and stages of RCC. These results suggest that DKK2 inhibits renal cancer progression through apoptotic and cell cycle pathways. (Clin Cancer Res 2009;15(18):5678–87)

Renal cell carcinoma (RCC) is the third leading cause of death among urological tumors, accounting for ~2% of adult malignancies (1). Although the rate of detection of incidental RCC has increased with improved diagnostic techniques, metastatic lesions are still found at diagnosis in ~30% of RCC patients (2). Wnt/β-catenin signaling is involved in renal cancer. Canonical Wnt ligands bind to frizzled (FZD) family receptors and the LRP5/LRP6 coreceptor, which stabilize β-catenin. Subsequently, β-catenin interacts with members of the lymphoid enhancer factor 1/T-cell factor family, resulting in generation of a functional transcription factor complex and the expression of downstream target genes (3, 4). Noncanonical Wnt ligands also bind to FZD family receptors and ROR2 and RYK coreceptors (4–7). This signaling is mainly involved in cytoskeletal reorganization during cancer cell invasion and metastasis (6, 7). At present, five Wnt antagonist families have been described, namely, secreted FZD-related protein (sFRP), Wnt inhibitory factor 1, Xenopus Cerberus, Wise, and Dickkopf (DKK) families (8).

Among Wnt antagonists, the DKK family consists of four main members (DKK1-DKK4), which contain two distinct cysteine-rich domains (3). DKK1 is typically silenced in colon cancer by hypermethylation, and its methylation status is

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Translational Relevance

DKK2, a Wnt antagonist, is silenced in some cancers, although its function has not been investigated in renal cancer. The CpG sites of the *DKK2* promoter region were methylated in renal cancer cell lines, and decreased levels of DKK2 were significantly increased after treatment with 5-Aza-2′-deoxycytidine alone or 5-Aza-2′-deoxycytidine and trichostatin A. Chromatin immunoprecipitation assays revealed histone modifications at the *DKK2* promoter region in renal cancer cell lines. In renal cell carcinoma tissues, expression of DKK2 was significantly lower than that of adjacent normal tissues, and the methylation status of the *DKK2* gene was higher in renal cancer tissues. There was a positive association between methylation status and higher grades, pathologic stages, and pathologic tumor in renal cancer. In functional studies, *DKK2* transfection inhibited renal cancer cell progression through apoptotic and cell cycle pathways. These results suggest that DKK2 is involved in renal cell carcinoma progression by regulating cell proliferation and apoptosis.

correlated with tumor progression in advanced stages of colon cancer (9). *DKK3* has also been reported to act as a tumor suppressor gene in various cancers, such as breast, pancreatic, cervical, non–small cell lung, bladder, prostate, renal, and leukemia (10–17). DKK2 is generally thought to be a direct inhibitor of Wnt binding to the LRP5/LRP6 coreceptors of FZD. In melanoma and gastrointestinal cancer, DKK2 expression is markedly decreased; however, the details of DKK2 function were not well investigated (18–20). Therefore, based on this literature, we hypothesized that (*a*) the mechanism of DKK2 down-regulation in renal cancer is through epigenetic alteration, including DNA methylation; (*b*) histone modification may also be involved in the silencing of DKK2 expression; (*c*) *DKK2* gene methylation status may correlate with clinicopathologic variants in renal cancer; (*d*) high DKK2 expression may contribute to inhibition of renal cancer cell proliferation via apoptosis and/or cell cycle arrest.

To test this hypothesis, we did methylation analysis of the *DKK2* gene promoter using renal cancer cell lines, a normal kidney cell line, and human renal cancer and adjacent normal tissue samples. We also investigated the relationship between the methylation frequency and clinical parameters, including tumor grade and pathologic stage. Chromatin immunoprecipitation (ChIP) analysis was carried out to assess histone modification at the *DKK2* gene promoter. We also monitored cell viability, colony formation, invasion, and apoptosis, and did cell cycle analysis, using transfected cells that overexpressed DKK2. We also investigated the molecular mechanisms involved in apoptosis and cell cycle–related genes via the canonical and noncanonical Wnt signaling pathways.

Materials and Methods

Cell culture. RCC cell lines (A498 and Caki2) and normal kidney cell line (HK-2) were purchased from the American Type Culture Collection. The A498 and Caki2 cell lines were incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and 10 mmol/L HEPES buffer. The HK-2 cell line was incubated with keratinocyte serum-free medium supplemented with 5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract.

Clinical samples. A total of 52 patients (36 male and 16 female) with pathologically confirmed conventional RCC were enrolled in this study (Toho University Hospital, Tokyo, Japan). The mean ages of the patients were 59.9 (range, 37-77; Supplementary Table S1). They were classified according to the WHO criteria and staged according to the tumor-node-metastasis classification. The pathology of all the patients was clear cell renal carcinoma.

DNA, RNA, and protein extraction. Genomic DNA was extracted from cell lines, human renal cancer, and adjacent noncancerous normal tissue using a QIAamp DNA Mini kit (Qiagen) after microdissection. RNA was extracted from cell lines using a QIAamp RNA kit (Qiagen). Cells were lysed with a CelLytic Extraction kit containing protease inhibitors (Sigma). Protein quantification was done using a bicinchoninic acid protein assay kit (Pierce).

Bisulfite DNA sequencing. Genomic DNA was modified with sodium bisulfate using a CpGenome Fast DNA modification kit (Chemicon International). The first universal primer sets (Seq PR1 and Seq CP2-1) cover no CpG sites in both forward and reverse primers and amplified a DNA fragment of the promoter region containing several CpG sites. Then, nested PCR was done using the first universal PCR products as templates. We did bisulfate DNA sequencing using the second PCR products. The primers used are shown in Supplementary Table S1.

5-Aza-2′-deoxycytidine and trichostatin A treatment. RCC cell lines (A498 and Caki2) were treated with the demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-dc; 1 μmol/L) alone for 14 d or treated with both 5-Aza-dc and histone deacetylase inhibitor trichostatin A (TSA; 500 nmol/L for 24 h; Upstate Biotechnology) in triplicate. In the combined treatment (5-Aza-dc and TSA), cells were treated with 1 μmol/L 5-Aza-dc for 14 d followed by 500 nmol/L TSA for an additional 24 h. Genomic DNA, total RNA, and protein were prepared from each cell line before and after treatment for sequencing, reverse transcription-PCR (RT-PCR; TITANIUM One-Step RT-PCR kit, BD Biosciences), and Western blot.

ChIP assay. ChIP assays were done on cell line DNA using an EZ CHIP kit (Upstate Biotechnology) according to the manufacturer's instructions. Chromatin was sonicated to obtain fragmented DNA (100-200 bp) and immunoprecipitated with antibody. Antibody for acetylated histone H3, acetylated histone H4, and dimethylated histone H3K4 was obtained from Upstate Biotechnology. Dimethylated histone H3K9 antibody was from Abcam. The primer pairs used for ChIP assays are shown in Supplementary Table S1. One additional primer set was used to amplify a 166-bp fragment of the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene as an internal control. Each PCR was initially set up using different amounts of ChIP sample with varying PCR cycle numbers to select the option PCR conditions. PCR products were analyzed on 2.0% agarose gels and visualized by staining with ethidium bromide. Densitometric analysis of the observed bands was done using ImageJ software.[4] Fold enrichment in each immunoprecipitation was determined by the ratio to input DNA. Two independent ChIP experiments were done for each analysis, and PCR was done twice for each of the ChIP-DNA samples.

Immunohistochemical study. Immunostaining of DKK2 and β-catenin was done in formalin-fixed, paraffin-embedded specimens using rabbit polyclonal antibody against human DKK2 (Abgent) and human β-catenin (Cell Signaling Technology). The staining procedure was according to a commercial kit (Lab Vision). The sections were counterstained with Harris' hematoxylin. Immunohistochemical staining was evaluated by assessing staining intensity (0-2) using a microscope at

[4] http://rsb.info.nih.gov/ij/

Table 1. DKK2 expression quantified in normal and RCC tissues

IHC score	Normal kidney *n* (%)	RCC (clear cell) *n* (%)
0	0 (0)	43 (80)
1	2 (4)	7 (14)
2	48 (96)	0 (0)
Total	50 (100)	50 (100)

200×. All specimens were scored blindly by two observers. The criteria of intensity are as follows: 0, negative expression; 1+, weakly positive expression; and 2+, strongly positive expression.

Plasmid construction. Plasmids containing the human full-length cDNA fragment of *DKK2* (Genbank accession number NM_014421) were purchased from Origene. This clone (pCMV6-DKK2) expresses the complete DKK2 open reading frame with a Tag (MYC/DDK) at the COOH terminus.

Stable clone establishment. To prepare stable cell lines overexpressing *DKK2*, we transfected A498 cells with the pCMV6-DKK2 expression vector encoding *DKK2* cDNA using FuGENE HD (Roche Diagnostics) according to the manufacturer's instructions. Transfected cells were selected by culturing with G418 (200 μg/mL) for 1 mo. Single colonies of stable transfectants were isolated and expanded for further analysis based on the level of DKK2 expression. Experiments were done with several independent clones to avoid clonal effects.

Cell viability assay. *DKK2* stably transfected A498 cells were maintained in medium supplemented with 200 μg/mL G418. Cell viability was measured after 7 d with MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega). Data are the mean ± SD of six independent experiments.

Soft agar colony formation assay. Soft agar colony formation was assayed with A498 mock cells and A498 *DKK2* stably transfected cells using a Cell Biolabs CytoSelect Cell Transformation Assay kit.

Namely, cells were incubated for 7 d in a semisolid agar medium before being solubilized and detected by using the provided MTT solution in a microplate reader (A_{570nm}). The absorbance was compared between mock and *DKK2*-transfected cells. Data are the mean ± SD of 10 independent experiments.

Cell invasion assay. Cell invasion assay was done with six-well BD Biocoat Matrigel invasion chambers as previously described (BD Biosciences). The cells (mock and A498 *DKK2*–transfected cells) were resuspended to the upper chamber in triplicate. Cells migrating through the membrane were stained with HEMA3 (Fisher Scientific Co.) and counted with a microscope. Five random fields were chosen for each membrane, and the results were expressed as migrated cells per field.

Apoptosis and cell cycle analysis. Cells (mock and *DKK2*-transfected cells) were trypsinized and washed once in complete medium centrifuged at 2,000 rpm for 5 min at 4°C and resuspended in ice-cold 1×

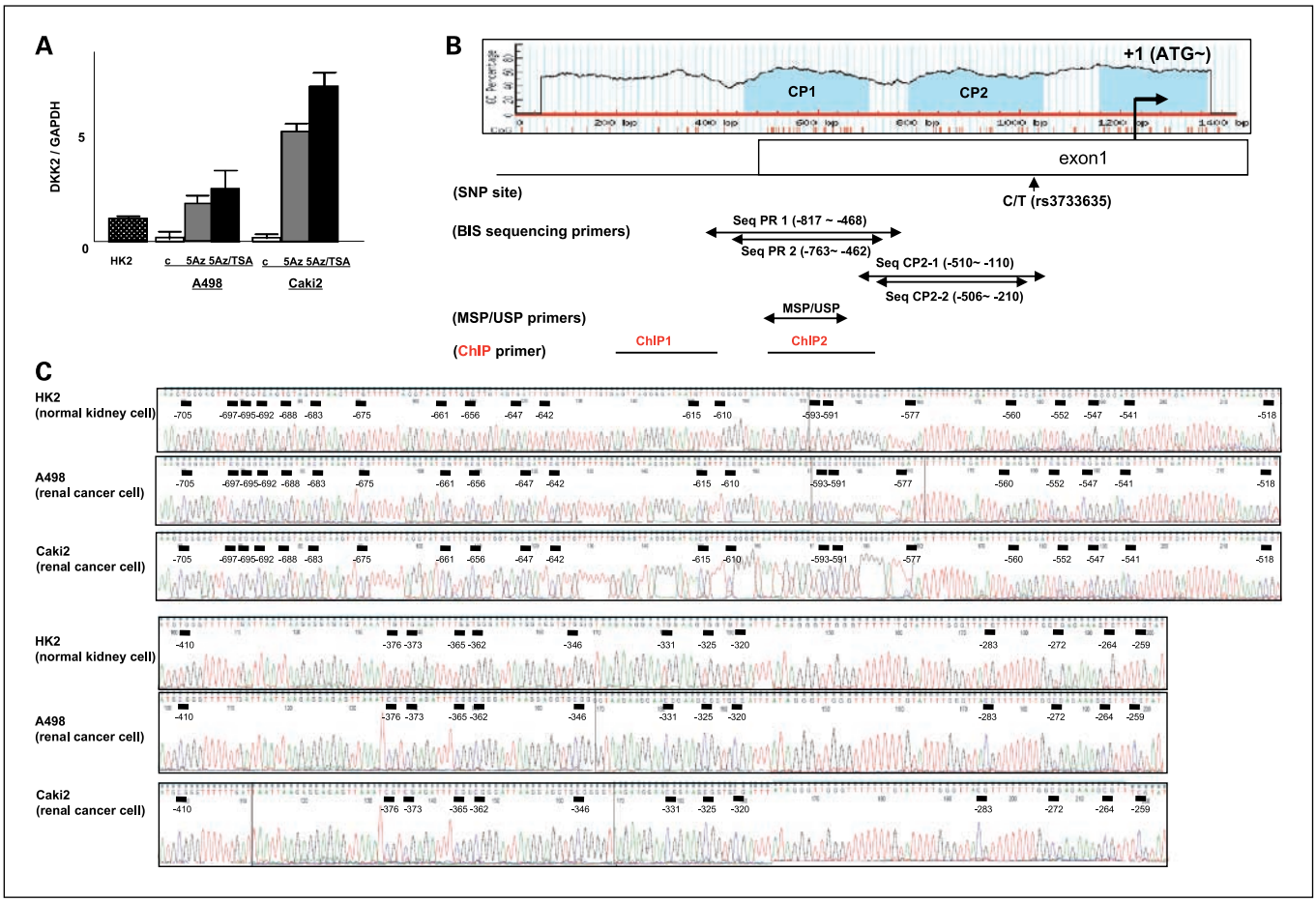

Fig. 1. Expression levels of DKK2 mRNA in normal kidney HK2 cells and renal cancer cell lines (with and without 5-Aza-dc/TSA treatment) and schematic representation of the location of CpG sites, primers, and methylation status in the functional promoter of the *DKK2* gene. *A,* expression levels of DKK2 mRNA in normal kidney cells (HK2) and renal cancer cell lines (A498 and Caki2). *B,* schematic representation of the location of CpG sites and primers in the functional promoter of the *DKK2* gene. *C,* representative bisulfite-modified genomic DNA sequencing of *DKK2* promoter region from normal kidney cell (HK2) and renal cancer cells (A498 and Caki2). The methylation status at 34 CpG sites of the 2 CpG islands, CP1 (21 CpG sites) and CP2 (13 CpG sites), was determined in these cell lines.

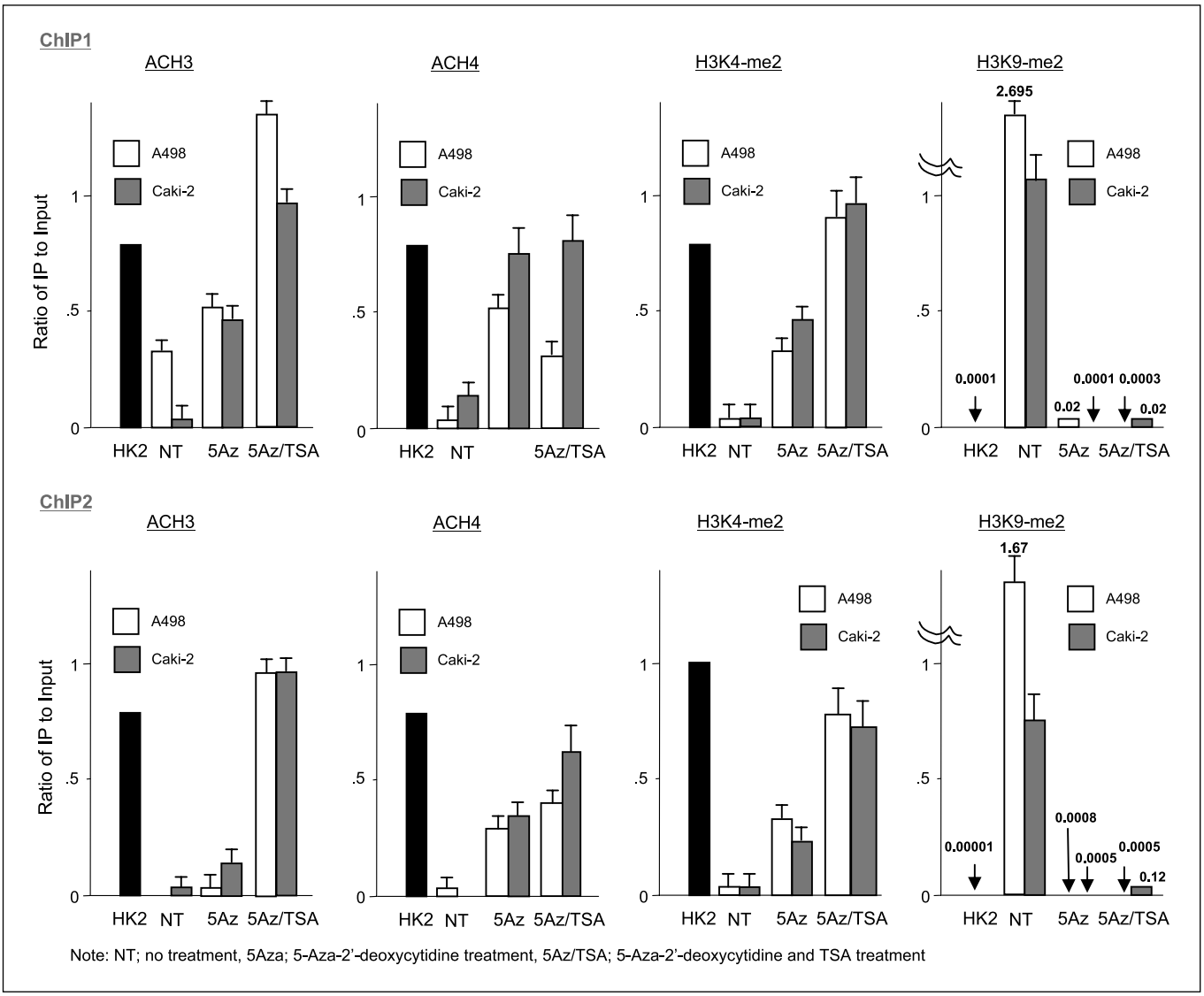

Fig. 2. Quantification of ChIP-PCR analysis in a normal kidney cell line (HK2) and renal cancer cell lines (A498 and Caki2). NT, no treatment; 5Az, 5-Aza-dc treatment; 5Az/TSA, 5-Aza-dc and TSA treatment.

binding buffer. Annexin V-FITC solution (10 μL) and 7-aminoactinomycin D viability dye (20 μL) were added to 100 μL of the cell suspensions. After incubation for 15 min in the dark, 400 μL of ice-cold 1× binding buffer were added. The apoptotic distribution of the cells in each sample was then determined using a fluorescence-activated cell sorter (Cell Lab Quanta SC, Beckman Coulter). The various phases of cells were determined using a DNA stain (4′,6-diamidino-2-phenylindole). Cell populations (G_0-G_1, S, and G_2-M) were measured using fluorescence and contrasted against cell volume. Data are the mean ± SD of four independent experiments.

Quantitative real-time RT-PCR in cell lines. Quantitative real-time RT-PCR was done in triplicate with an Applied Biosystems Prism 7500 Fast Sequence Detection System using Taqman Universal PCR master mix according to the manufacturer's protocol (Applied Biosystems, Inc.). The Taqman probes and primers were purchased from Applied Biosystems. Human GAPDH was used as an endogenous control. Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems).

Western blotting. Total protein (20 μg) was used for Western blotting. Samples were resolved in 4% to 20% Precise Protein Gels (Pierce) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The membranes were immersed in 0.3% skim milk in TBS containing 0.1% Tween 20 for 1 h and probed with primary polyclonal and monoclonal antibody against β-catenin, Bcl2, Bax, FADD, Bid, caspase-3, cytochrome *c*, cyclin D1, c-Jun NH_2-terminal kinase (JNK), phospho-JNK, c-Jun, phospho–c-Jun, and GAPDH (14C10; Cell Signaling Technology) overnight at 4°C. To confirm DKK2 stable transfectant, we used anti-DKK2 antibody (Cell Signaling Technology). Blots were washed in TBS containing 0.1% Tween 20 and labeled with horseradish peroxidase–conjugated secondary anti-mouse or anti-rabbit antibody (Cell Signaling Technology). Proteins were enhanced by chemiluminescence (ECL Plus Western Blotting Detection System, Amersham Biosciences) for visualization. The protein expression levels were expressed relative to GAPDH levels.

Statistical analysis. All statistical analyses were done using StatView (version 5; SAS Institute, Inc.). A P value of <0.05 was regarded as statistically significant.

Results

WNT antagonist gene expression. We examined the RNA expression of all DKKs and sFRPs in a RCC cell line (A498) and

compared it with normal kidney cell (HK2). Among them, sFRP4 expression was significantly higher in renal cancer cells compared with the normal kidney cells (HK2).

In contrast, the mRNA expression levels were significantly lower for DKK2, DKK3, DKK4, sFRP1, sFRP2, sFRP3, and sFRP5 compared with normal kidney cell (HK2; Supplementary Fig. S1). Given that the details of DKK2 function are currently not understood (18–20), we focused this investigation on the role of DKK2 in renal cancer.

DKK2 expression in RCC cell lines before and after treatment with 5-Aza-dc and TSA. DKK2 expression of all kidney cancer cell lines was lower compared with that of the normal kidney cell line (HK2). After treatment with only 5-Aza-dc or both 5-Aza-dc and TSA, the expression of DKK2 was significantly increased (Fig. 1A).

Methylation status of the DKK2 gene promoter region in cell lines. We analyzed the *DKK2* gene promoter CpG islands using bisulfite-modified genomic DNA from A498, Caki2, and HK2 cell lines. The methylation status at 34 CpG sites of the 2 CpG islands, CP1 (21 CpG) and CP2 (13 CpG), was determined in these cell lines (Fig. 1B). The detailed CpG methylation patterns are shown in Supplementary Fig. S2A. The CpG sites of the *DKK2* gene promoter were not methylated in HK2 cells. However, the CpG sites of the *DKK2* promoter were almost completely methylated in renal cancer cell lines (A498 and Caki2). We also did methylation-specific PCR (MSP) and unmethylated-specific PCR (USP) in DKK2 promoter CpG sites in renal cancer cell lines and normal kidney HK2 cells. We observed only USP and MSP bands in HK2 and renal cancer cells, respectively (Supplementary Fig. S2B). Representative bisulfite sequences in normal kidney (HK2) and renal cancer cell lines (A498 and Caki2) are shown in Fig. 1C.

Histone modification associated with the DKK2 gene promoter region. ChIP assays were done to determine whether epigenetic suppression of *DKK2* was caused by local histone acetylation (H3 and H4) and H3 methylation in the chromatin associated with the *DKK2* promoter region. The histone-associated DNAs, immunoprecipitated with antibodies including acetylated histone H3, acetylated histone H4, H3K4-me2, and H3K9-me2, were amplified with two primer sets (ChIP1 and ChIP2) covering the *DKK2* promoter region (Fig. 2). Results of ChIP assay are shown in Fig. 2 and Supplementary Fig. S3.

In HK2 cells (normal kidney cell line), whose promoter region is unmethylated in the *DKK2* gene and whose expression is very high, levels of acetyl H3, acetyl H4, and dimethyl H3K4 were high, whereas the level of dimethylated H3K9 was low. In contrast, levels of acetyl H3, acetyl H4, and dimethyl H3K4 were very low and the level of dimethylated H3K9 was very high in the renal cancer cell lines (A498 and Caki2). Histone modifications in cancer cells were similar to those of the normal kidney cell line after 5-Aza-dc and TSA treatment (Fig. 2).

DKK2 expression in human normal kidney and cancer tissues. Based on the methylation and ChIP assay results in cell lines, we did an immunohistochemical study using clinical samples (50 RCC and 50 adjacent normal tissues). All samples were clear cell carcinoma. The expression of DKK2 was significantly lower in RCC tissues (clear cell carcinoma) than in adjacent normal tissues (Fig. 3 and Table 1).

The relationship between DKK2 methylation status and clinicopathologic factors. We assessed the methylation status of the

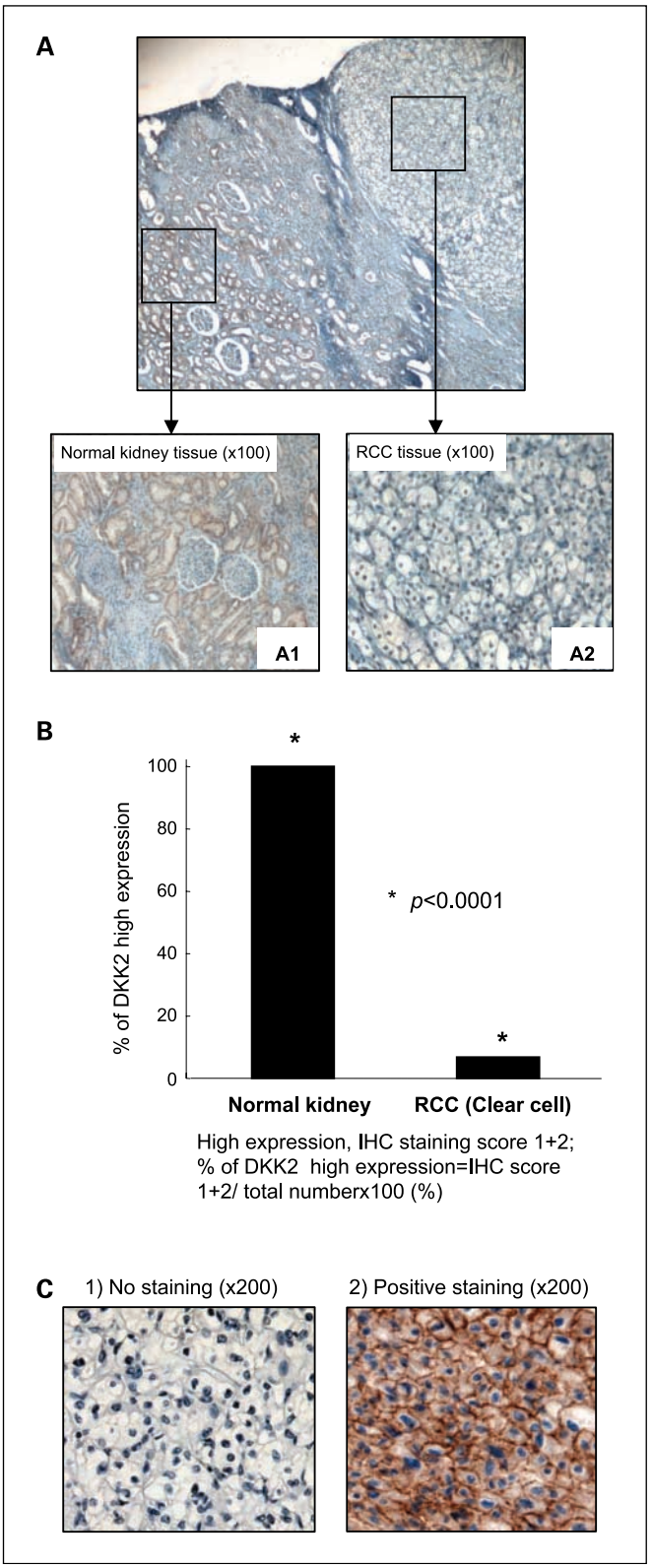

Fig. 3. Immunohistochemistry of DKK2 and β-catenin expression in human renal cancer tissues and adjacent normal kidney tissues from the same RCC patients. *A,* representative immunostaining of DKK2 in human normal kidney (*A1*) and RCC (clear cell carcinoma) samples (*A2*). *B,* DKK2 expression was quantified in normal and RCC tissues. Summary of immunostaining data. *C,* representative immunohistochemical staining of β-catenin in RCC tissues: 1, no staining; 2, positive staining.

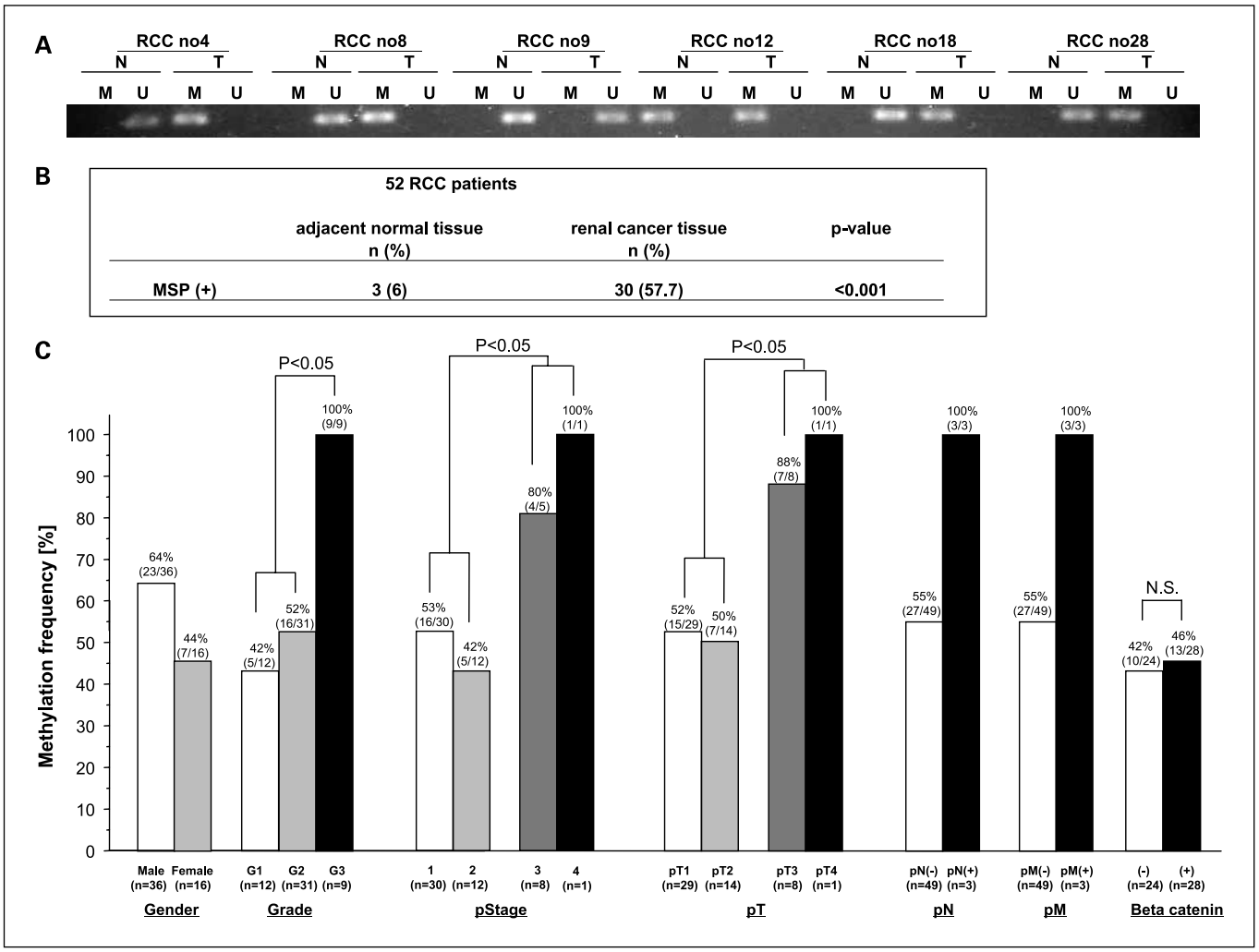

Fig. 4. Methylation status of the *DKK2* gene promoter region and correlation with clinicopathologic parameters in primary RCC samples. *A,* representative MSP and USP in primary RCC tissues (*T*) and adjacent normal kidney tissues (*N*) from the same patients. *B,* summary of MSP in renal cancer tissues and adjacent normal kidney tissues in 52 RCC patients. *C,* correlation of *DKK2* gene promoter methylation frequency with clinicopathologic parameters in RCC patients. Clinicopathologic parameters included gender, grade, pathologic stage (*pStage*), pathologic tumor-node-metastasis, and β-catenin expression.

DKK2 gene in clinical samples and found that methylation of the *DKK2* gene was significantly higher (30 of 52, 57.7%) in renal cancer tissues than in adjacent normal tissues (3 of 52, 6%; $P < 0.01$; Fig. 4A and B). We also looked at methylation frequency according to various parameters, such as gender (male, female), grade (1, 2, and 3), pathologic stage (pStage1, pStage2, pStage3, pStage4), pathologic tumor (pT1, pT2, pT3, pT4), pathologic node [pN(-), pN(+)], and pathologic metastasis [pM(-), pM(+); Fig. 4C].

The methylation frequency was significantly higher in higher grades (grade 3+4), pathologic stages (pStage3+4), and pathologic tumor (pT3+pT4) of renal cancer (Fig. 4C).

The methylation frequency was also higher in patients with pN(+) or pM(+) (Fig. 4C).

We did not find a significant relationship between β-catenin expression and methylation status of the *DKK2* gene (Fig. 4C).

Effect of DKK2 on cell viability, colony formation, and cell invasion. After transfection of A498 cells with a pCMV6-DKK2–expressing plasmid, several individual clones (no. 1-5) were tested for DKK2 mRNA and protein expression by real-time RT-PCR and Western blotting (Fig. 5A). Little is known about the function of DKK2 *in vitro*. Therefore, we did cell viability analysis (MTS assay), colony-forming assays, and cell invasion assays using stably transfected A498 cells that overexpressed DKK2. At day 7, the number of A498 cancer cells was significantly decreased in *DKK2*-transfected cells compared with mock cells (Fig. 5B, *1*). The number of colonies was also significantly decreased in *DKK2*-transfected cells compared with mock cells (Fig. 5B, *2*). However, DKK2 did not affect the *in vitro* invasion ability of A498 (data not shown).

Apoptosis and cell cycle analyses. Next, we did apoptosis and cell cycle analysis to investigate whether DKK2 overexpression affects these parameters in renal cancer cells. We found a significantly higher number of apoptotic cells in *DKK2*-transfected cells compared with mock cells ($P < 0.001$; Fig. 5C). The mean cell number in the S and G_2-M phases of the cell cycle was also significantly lower in *DKK2*-transfected cells, suggesting that DKK2 induced G_1 arrest in A498 cells (Fig. 5C).

Quantitative real-time RT-PCR and Western blotting in DKK2-overexpressed cells. We found growth inhibition and increased

apoptosis in *DKK2*-transfected cells, suggesting that DKK2 affects these functions in renal cancer cells. Therefore, we focused our study on apoptosis and cell cycle–related genes. Among these candidate genes, Bcl2 RNA expression in DKK2-overexpressing cells was significantly lower than that in mock cells ($P < 0.01$; Fig. 6A, *1*). In contrast, Bax, Bid, and FADD RNA expression in *DKK2*-transfected cells was significantly higher than in mock cells (Fig. 6A, *2-4*). These results were confirmed by Western blotting. Expression of Bcl2 and Bax protein was significantly down-regulated and up-regulated, respectively, in *DKK2*-transfected cells, whereas there was no significant difference in the expression level of Bid and FADD (Fig. 6B). Unexpectedly, there was no difference in caspase-3, cytochrome *c*, and β-catenin expression between mock and *DKK2*-transfected cells. However, cyclin D1 expression was dramatically decreased in *DKK2*-transfected cells (Fig. 6B). About the levels of JNK pathway-related proteins, there was no difference between mock and *DKK2*-transfected cells (data not shown).

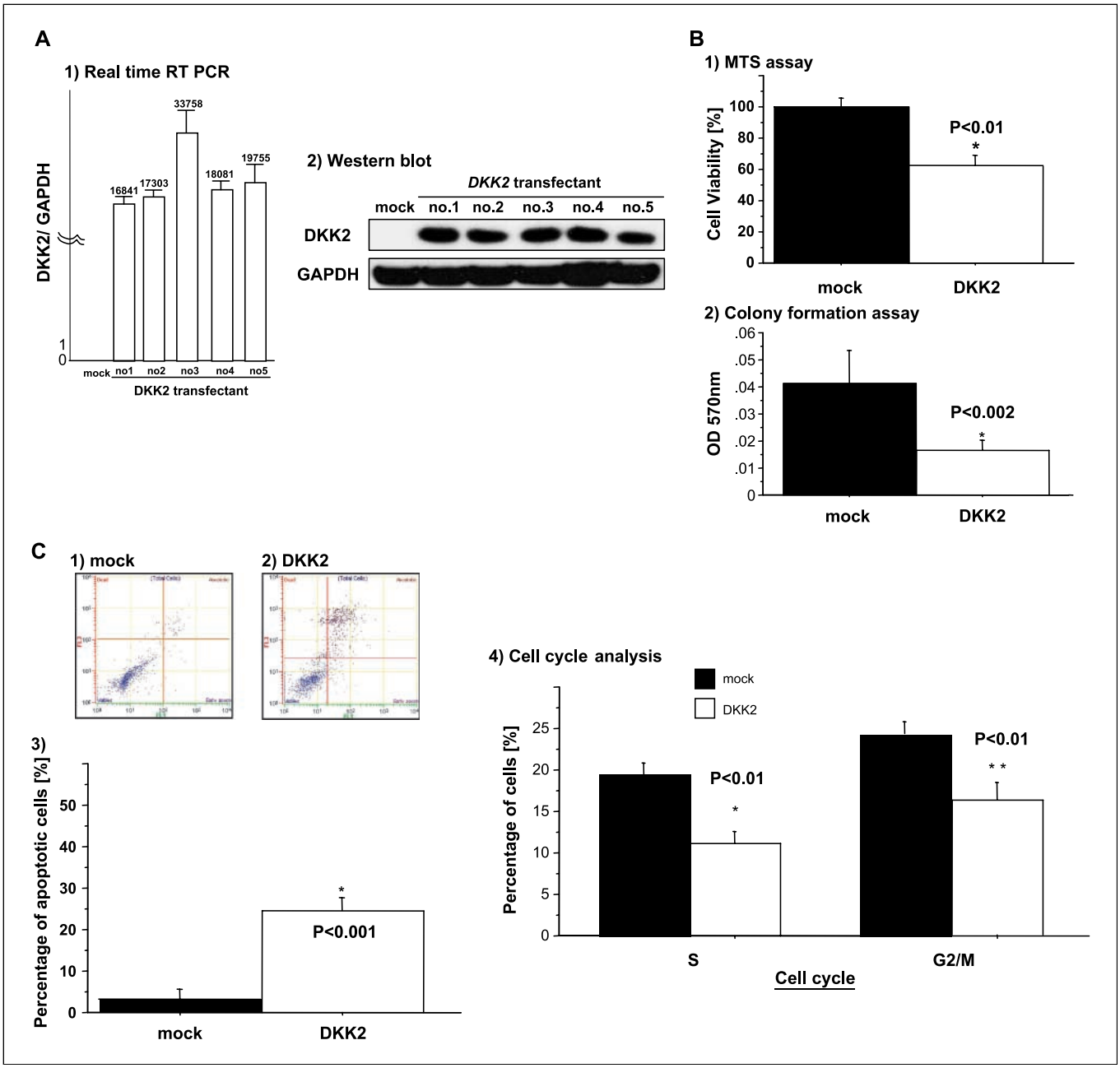

Fig. 5. Comparison of cell viability, colony formation, apoptosis, and cell cycle analysis in mock and *DKK2*-transfected A498 cells. *A,* expression of DKK2 in stable transfectants: 1, real-time RT-PCR; 2, Western blots. *B,* cell viability and colony formation assay in mock and *DKK2*-transfected cells. *C,* flow cytometry analysis of apoptosis and cell cycle in mock and *DKK2*-transfected A498 cells. Annexin V-FITC and 7-aminoactinomycin D were measured by flow cytometry. 1 and 2, representative results. 3, columns, mean of four independent experiments; bars, SD. Enumeration of the various phases of the cells was determined using a DNA stain, such as 4′,6-diamidino-2-phenylindole. G_0-G_1, S, and G_2-M populations were measured using fluorescence and contrasted against cell volume. 4, columns, mean of four independent experiments; bars, SD.

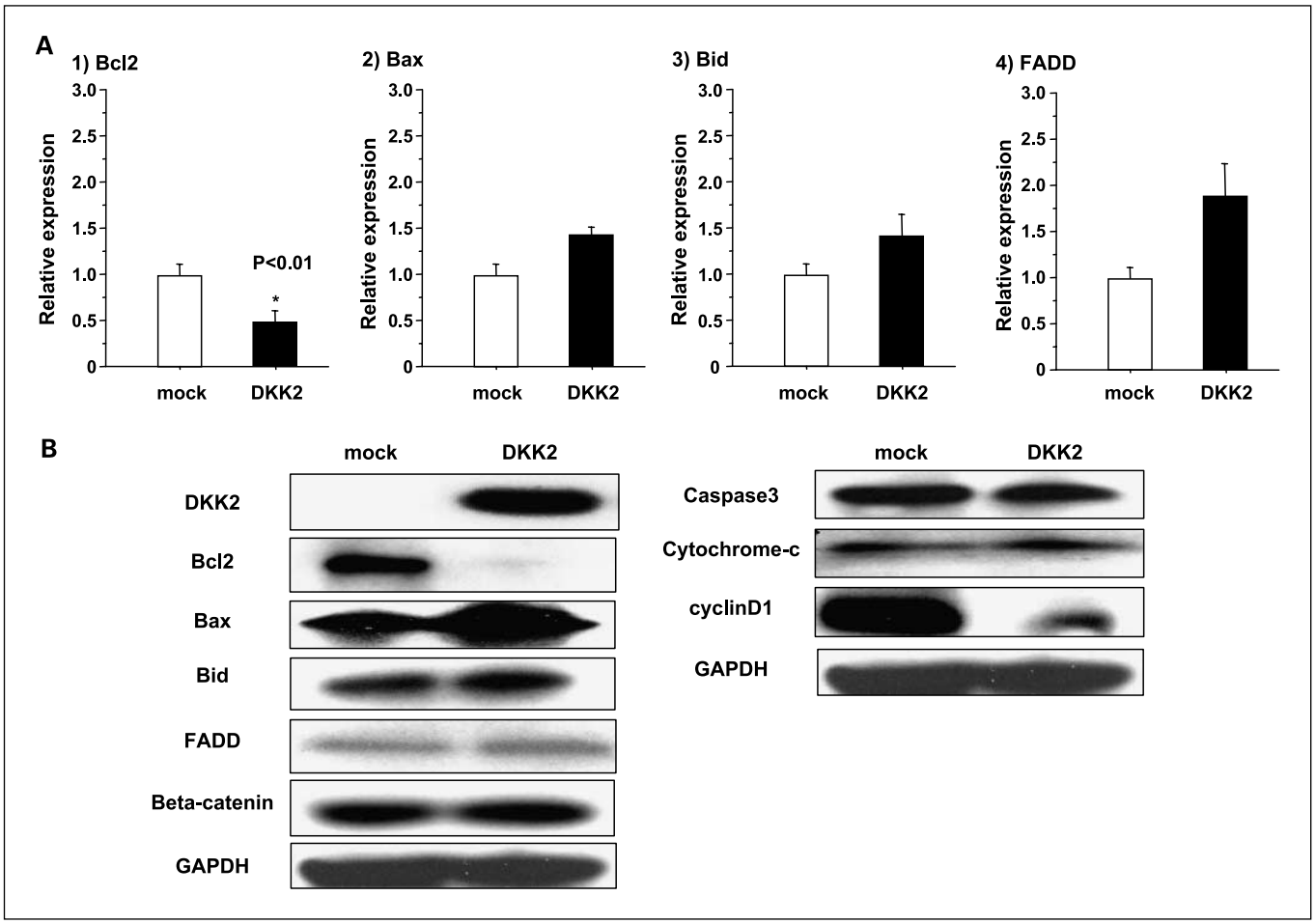

Fig. 6. Apoptosis and cell cycle gene mRNA and protein expression level in mock and *DKK2*-transfected A498 cells. *A,* apoptosis and cell cycle gene mRNA expression in mock and *DKK2*-transfected A498 cells. *B,* apoptosis and cell cycle gene protein expression in mock and *DKK2*-transfected A498 cells.

Discussion

In this study, we found that silencing of DKK2 is caused by methylation and histone modification of the *DKK2* gene promoter region in renal cancer cell lines. We previously showed that histone modification was involved in silencing the *sFRP2* gene in renal cancer (21). Recently, Lee et al. (22) found that DKK1 was repressed by histone deacetylation in cervical cancer cells; however, until now, there have been no reports about histone modifications of the *DKK2* gene and renal cancer. Therefore, we used ChIP assay to examine the role of this silencing mechanism in DKK2 expression in renal cancer cell lines and we found that histone acetylation and H3K4 methylation were increased in normal kidney cell line (HK2), resulting in an open chromatin structure associated with active gene expression. In contrast, renal cancer cell lines (A498 and Caki2) had a different histone structure. In addition to H3K9, which is regarded as a repressive histone modification indicative of gene silencing (23, 24), the promoter region was also highly methylated in renal cancer cells (A498 and Caki2), whereas there was no methylation in normal kidney HK2 cells. After treatment with DNA methyltransferase inhibitor (5-Aza-dc) and/or a histone deacetylase inhibitor (TSA), histone patterns (acetyl H3, acetyl H4, dimethyl H3K4, and dimethyl H3K9) in renal cancer cells were similar to those of the normal kidney cell line HK2. Thus, down-regulation of DKK2 associated with repressive histone modifications may also be involved in the silencing of DKK2 expression in renal cancer cells.

We also observed that DKK2 expression was clearly decreased in renal cancer tissues, and in accordance with this result, the methylation frequency was higher in renal cancer tissues compared with adjacent normal kidney tissues. In addition, we found that higher methylation frequency was significantly associated with high grades (grade 3+4), pathologic stages (pStage3+4), and pathologic tumor (pT3+pT4) in renal cancer. These results are consistent with previous reports in gastrointestinal cancer (19, 20).

DKK3, a member of the DKK family, has been reported to be associated with the noncanonical Wnt pathway in various cancers (25–28) and has also been reported to be involved in the canonical pathway in others (12, 29). Several studies have indicated that DKK3 functions as a tumor suppressor for human cancer growth and is involved in apoptosis (26–29). In contrast, the function and biological role of DKK2 is currently not understood. However, the relationship between methylation status and clinical factors suggests that DKK2 may be involved in cell survival or proliferation. We investigated the role of DKK2 in cell viability, colony formation, cell invasion, apoptosis, and the cell cycle using renal

cancer cells that overexpressed DKK2. In these studies, we found that there was a significant increase in inhibition of cell growth and induction of apoptosis in DKK2-overexpressing cells. These results are consistent with the study of Sato et al. (19) of DKK2 in gastrointestinal cancer cells. For instance, the cell population in the S and G_2-M phases decreased in *DKK2*-transfected cells, suggesting that DKK2 induced G_1 arrest in A498 cells.

To investigate in more detail the mechanism of the effect of DKK2 on apoptosis and the cell cycle, we evaluated the expression of apoptosis and cell cycle–related genes. Among these genes, Bcl2 expression was significantly down-regulated and Bax expression was slightly up-regulated in *DKK2*-transfected cells. Cyclin D1 expression was also significantly down-regulated in *DKK2* transfectants. However, caspase-3 expression was not altered after *DKK2* transfection. Bcl2, an anti-apoptosis protein, is essential for apoptosis (30). The apoptosis cascade also includes caspase-dependent and caspase-independent pathways (31), and loss of Bcl2 was in a caspase-independent manner in renal cancer cells (32). Cyclin D1 activates G_1-S transition of the cell cycle. It has been previously shown that cyclin D1 is overexpressed in renal cancer cells (33). Recently, Gumz et al. (34) reported that cyclin D1 was up-regulated in clear cell RCC, and one of the Wnt antagonists, sFRP1, inhibited cyclin D1 expression. Our results show a similar effect of DKK2 on cyclin D1. Thus, accumulating evidence suggests that DKK2 plays a role in apoptosis and inhibits renal cancer proliferation by targeting Bcl2 and cyclin D1. However, it is currently unknown whether DKK2 directly affects Bcl2 or whether other genes are also involved in this signal transduction.

We also looked at β-catenin expression using immunohistochemical techniques to assess the relationship between *DKK2* gene methylation status and β-catenin expression. Based on previous data, we expected that high methylation of the *DKK2* gene might correlate with high β-catenin expression. However, we found no significant relationship between *DKK2* gene methylation and β-catenin expression. We also unexpectedly found that DKK2-overexpressing cells did not affect β-catenin expression. DKK2 is generally regarded as a direct inhibitor of the Wnt canonical pathway. To test whether DKK2 also is involved in the noncanonical pathway, we looked at the JNK pathway, which is a part of noncanonical Wnt signaling. In mock and *DKK2*-transfected cells, we found no difference in expression of phospho-JNK, c-Jun, and phospho–c-Jun (data not shown). Although we did not examine all the noncanonical pathways, such as NF-κB signaling, no significant difference was seen in invasive capability between mock and *DKK2*-transfected cells. The noncanonical Wnt pathway is generally regarded as an important feature of cancer metastasis and invasion (35). Thus, our results suggest that DKK2 is primarily involved in the Wnt canonical pathway.

In conclusion, this is the first report to show that DKK2 expression is epigenetically silenced in RCC and that the methylation frequency of the *DKK2* gene promoter region is higher in RCC patients with higher grades and stages of the disease. Furthermore, our results suggest that DKK2 plays a role in apoptosis and cell proliferation by controlling levels of Bcl2 and cyclin D1 in a caspase-independent manner.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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