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

Hiroshi Hirata,1 Yuji Hinoda,2 Koichi Nakajima,3 Ken Kawamoto,1 Nobuyuki Kikuno,1 Kazumori Kawakami,1 Soichiro Yamamura,1 Koji Ueno,1 Shahana Majid,1 Sharanjot Saini,1 Nobuhisa Ishii,3 and Rajvir Dahiya1

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/G2-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...
DKK2 Inhibits RCC Progression through Apoptosis

**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 LRPs five-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 Col-
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 (A570nm). 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 in 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×

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**Table 1. DKK2 expression quantified in normal and RCC tissues**

<table>
<thead>
<tr>
<th>IHC score</th>
<th>Normal kidney n (%)</th>
<th>RCC (clear cell) n (%)</th>
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<tr>
<td>Total</td>
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**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.
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 (G0-G1, S, and G2-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 NH2-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 unmethylation-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 clinico-pathologic factors.** We assessed the methylation status of the
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 \( \beta \)-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 G2-M phases of the cell cycle was also significantly lower in DKK2-transfected cells, suggesting that DKK2 induced G1 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. G0-G1, S, and G2-M populations were measured using fluorescence and contrasted against cell volume. 4, columns, mean of four independent experiments; bars, SD.
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 has been 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 G2-M phases decreased in DKK2-transfected cells, suggesting that DKK2 induced G1 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 slightly up-regulated in these genes, Bcl2 expression was significantly down-regulated in 

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 G1-S transition of the cell cycle. It has been previously shown that cyclin D1 is overexpressed in renal cancer cells (33). Recently, Gunz 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.

Acknowledgments

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References

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