Epigenetic silencing of DKK2 and Wnt signal pathway components in human ovarian carcinoma

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Wnt/β-Catenin signaling dysregulation is involved in tumorigenesis. Furthermore, epigenetic modification of the Dickkopf (DKK) family (DKK1–DKK4) has been shown to be important in Wnt signaling regulation. In this study, the role of DKK2, a Wnt antagonist, in epithelial ovarian cancer (EOC) was examined by evaluating the expression and methylation of DKK2 in SKOV3 and ES-2 ovarian cancer cell lines and 78 tissues collected from patients (50 ovarian carcinoma, 20 benign tumor and 8 normal ovarian tissues). DKK2 is highly downregulated in EOCs; however, DKK2 expression levels are higher in both normal tissues and benign tumors. In most cases of ovarian carcinoma, DKK2 is methylated, compared with the more common unmethylated form present in benign tumors and normal ovarian tissues. Additionally, DKK2 may be epigenetically silenced by methylation in higher grades and stages of EOC. Functional analysis revealed that overexpression of DKK2 suppressed malignant cell growth and invasion in SKOV3 and ES-2 cell lines. The expression of the downstream genes of Wnt signaling, including β-catenin, c-Myc and cyclin D1, was decreased in DKK2-transfected cells compared with mock cells. The expression of matrix metalloproteinase-2 and focal adhesion kinase were also decreased in DKK2 transfectants, supporting findings indicating inhibition of cell migration and invasion. This report provides novel indications that DKK2 is a unique hypermethylated target gene in EOC and that DKK2 may contribute to tumorigenesis in EOC through the Wnt/β-catenin signaling mechanisms.

Introduction

Ovarian cancer is a common gynecological malignancy and one of the leading causes of cancer-related morbidity and mortality in female patients around the globe. In order to more effectively treat and prevent ovarian cancer, gynecological researchers face the challenge of developing a full understanding of the molecular events leading to initiation and progression of the disease. The Wnt signaling transduction pathway is known to play an important role in both embryonic development and tumorigenesis (1–3). Recent studies have revealed that Wnt signaling also plays a key role in the embryonic development of the ovary, and it is involved in normal follicular development and ovarian function (4,5). Recent research has also implicated Wnt signaling in human breast, colon, lung and ovarian cancer progression and metastasis (6–10). Although the mechanisms responsible for this effect are not fully understood, Wnt may be a key future therapeutic target in epithelial cell tumorigenesis.

There are at least three different Wnt pathways: the canonical pathway, the planar cell polarity pathway and the Wnt/Ca2+ pathway (11). In the canonical Wnt pathway, the major effect of Wnt ligand binding is the stabilization of cytoplasmic β-catenin through inhibition of the β-catenin degradation complex. β-Catenin is then free to enter the nucleus and activate Wnt-regulated genes by interaction with the T-cell factor (TCF) family transcription factors and concomitant recruitment of coactivators. Initiation of Wnt signaling is primarily modulated by the Wnt antagonists of the Wnt inhibitory factor 1, secreted FZD-related protein, Xenopus cerberus, Wise and Dickkopf (DKK) families (12–14). Members of the DKK protein family (DKK-1, -2, -3 and -4) are secreted proteins with two cysteine-rich domains separated by a linking region. DKK-3 and -4 also have one prokine- tin domain. DKK-1, -2 and -4 function as antagonists of canonical Wnt signaling by binding to LRP5/6 (LRP5/6) and thus preventing the interaction of LRP5/6 with Wnt-Frizzled complexes. DKK-1, -2 and -4 also bind cell surface kremen-1 or -2 and promote the internalization of LRP5/6 (15–18). Unlike other DKK family members that bind to TCF coreceptors, DKK3 seemed to inhibit Wnt/β-catenin signaling by preventing its nuclear translocation (19,20). DKK2 is a putative Wnt signaling inhibitor that is generally downregulated in human cancers, including renal clear cell carcinoma, melanoma, colorectal cancer and gastrointestinal tumors (7,21,22). It does, however, have notable functions as a putative tumor-suppressive molecule. In addition, kremen-2 can function as a switch that changes DKK2 from an activator to an inhibitor of Wnt/LRP6 signaling, potentially providing a regulatory pathway for Wnt action (18).

Conflicting data have been reported regarding DKK2 upregulation in a number of human cancers, including human colorectal cancer (8). These findings indicate that the expression of DKK2 in ovarian cancer, a previously unexplored target, may have significance in future treatment and prevention of the disease. This study investigates the expression of DKK2 in ovarian carcinomas and normal ovarian tissues in order to explore possible associations between DKK2 and Wnt signaling in the ovarian tumorigenesis. Furthermore, this study adds to the body of knowledge pertaining to DKK2 function and mechanism, which has been poorly documented despite increasing evidence of the potential importance of DKK2 in cancer research and treatment.

Materials and methods

Cell lines and tumor specimens

The human epithelial ovarian cancer (EOC) cell lines (SKOV3 and ES-2) were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and penicillin/streptomycin (1:100, Sigma). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. For analysis of DKK2 gene restoration, SKOV3 and ES-2 cells were treated with 5-aza-2′-deoxycytidine (5-aza-CdR) (0.1–10 μM) (Sigma) for 72 h. The drug and medium were replaced every 24 h. Cells were used between passages 6–10 and not later than 3 days after trypsinization.

A total of 78 tissue specimens were collected from surgical patients enrolled in the study, including 50 primary EOC (PEOC) tissues, 20 benign ovarian tumor tissues and 8 normal ovarian tissues. All specimens were obtained during surgery (Department of Obstetrics and Gynecology, Ren-Ji Hospital, Shanghai Jiao Tong University School of Medicine, China), frozen immediately in liquid nitrogen and stored at −80°C until analysis. Among the 50 PEOC tissue specimens, 30 tumors were determined to be stage I–II, and 20...
tumors were determined to be stage III–IV. All neoplasms involved primary ovarian tumors, and no patient received chemotherapy prior to the surgical operation.

**Quantitative real-time reverse transcriptase PCR**

RNA was extracted from cell lines and tissue samples using an RNaseasy® Mini Kit (Qiagen) and total RNA was reverse transcribed with a PrimeScript® RT reagent Kit (TaKaRa, China) according to the instructions provided by the manufacturer. Resultant complementary DNAs (cDNAs) underwent quantitative real-time reverse transcriptase PCR using an SYBR Green PCR Master Mix Reagent Kit (TaKaRa) according to the manufacturer’s protocol. PCR amplification was performed on an ABI7500 real-time system (Applied Biosystems). The primers for DKK2 were AGAACCGCTGGAATATG (forward) and GAAATGACGAGCACAGCAAA (reverse). The primers for glyceraldehyde 3-phosphate dehydrogenase, serving as the endog- enous controls, were ACCACATCTCCACCTTGG (forward) and CTGTTGCTGTAACCAAATCCTG (reverse). SDS s.v.1.4 (Applied Biosystems) software was used to perform comparative delta cycle threshold (Ct) analysis. To minimize random variation, each PCR experiment was performed in triplicate.

**Methylation analysis**

Genomic DNA was extracted from cell lines and tissue samples using a QIAamp DNA mini kit (Qiagen). Bisulfate treatment of genomic DNA was performed using an EpiTect bisulfite kit (Qiagen). Methylation was analyzed through the use of a methylation-specific PCR (MSP). PCR was conducted on 50 ng samples containing 50 ng bisulfite-treated DNA, 1 × Ex Taq buffer (Mg²⁺ Plus), 0.2 mM deoxynucleoside triphosphate mixture, 0.2 μM of each primer and 1.25 U Ex Taq HS (TaKaRa). The PCR protocol for MSP required: 35 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C, and a 5 min final extension at 72°C. MSP primers for unmethylated DKK2 were 5'-ATAAAAAATCAAAAAACATCCCCAAACCA-3' (forward) and 5'-ATAAAAAATCAAAAAACAATCCCCCAAAACCA-3' (reverse). MSP primers for methylated DKK2 were 5'-TTTTTGTATGCGTTCGTTTATGTCGT-3' (forward) and 5'-AAATAAAAATCAAAAAACATCCCCCAAAACCA-3' (reverse). MSP primers for methylated DKK2 were 5'-ATAAAAAATCAAAAAACATCCCCAAACCA-3' (forward) and 5'-ATAAAAAATCAAAAAACAATCCCCCAAAACCA-3' (reverse). MSP primers for methylated DKK2 were 5'-TTTTTGTATGCGTTCGTTTATGTCGT-3' (forward) and 5'-AAATAAAAATCAAAAAACATCCCCCAAAACCA-3' (reverse). MSP primers for methylated DKK2 were 5'-ATAAAAAATCAAAAAACATCCCCAAACCA-3' (forward) and 5'-ATAAAAAATCAAAAAACAATCCCCCAAAACCA-3' (reverse). MSP primers for methylated DKK2 were 5'-TTTTTGTATGCGTTCGTTTATGTCGT-3' (forward) and 5'-AAATAAAAATCAAAAAACATCCCCCAAAACCA-3' (reverse).

**Immunohistochemical study**

DDK2 immunostaining was completed using formalin-fixed, paraffin-embedded specimens with rabbit polyclonal antibody against human DKK2 (Abgent). Tumor sections were deparaffinized using xylene, rehydrated in a graded ethanol series and finally washed in deionized water. Antigen retrieval was completed in 10 mM citrate buffer (pH 6.0) in a microwave near the boiling stage for 10 min. Each section was blocked with 100–400 μl TBST/5% normal goat serum at 1 h at room temperature. Sections were incubated with rabbit polyclonal antibody against human DKK2 overnight at 4°C in a humidity chamber. Normal rabbit immunoglobulins were substituted for primary antibody as negative controls. Sections were then incubated with biotinylated antirabbit immunoglobulin for 1 h at 37°C followed by treatment with 3,3' diaminobenzidine (DAB; Sigma) working solution and counterstaining with hematoxylin and eosin. Immunohistochemical staining was evaluated by visual assessment of the staining extent using an optical microscope (Leica DM2000, Wetzlar, Germany) at ×200 magnification. All specimens were blindly scored by two observers. The extent of staining was recorded according to the following scale: 0 (<25%), 1 (25–50%), 2 (50–75%) or 3 (>75%) based on the percent of positive tumor cells observed.

**Stable transfection of ovarian cancer cells with DKK2**

Plasmids containing human full-length DKK2 cDNA were purchased from OriGene (Rockville, MD). The clone (pCMV6-DKK2) expressed the complete DKK2 open reading frame with a Tag (MYCIDKK) at the COOH terminus. SKOV3 and ES-2 cells were transfected with the pCMV6-DKK2 expression vector encoding DKK2 cDNA using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. To establish the cells stably expressing DKK2, transfected cells were selected by culturing in G418 (400 μg/ml) for 10 days. Single colonies of stable transfectants were isolated and expanded for further analysis. Experiments were completed using several independent clones to avoid error due to clonal effects. Similarly, a reference clone transfected only with the vector (pCMV6) was used as the mock clone. Vector-only transfecants and parental cells (SKOV3 and ES-2 cells) were used as controls in the following experiments.

**β-Catenin/TCF report assay**

Cells were plated at 1 × 10⁵ cells/well on 96-well plates 24 h before transfection, and each well was subsequently transfected with 200 ng of a luciferase reporter plasmid containing wild-type or mutated TCF-binding sites, 50 ng of pcDNA-DKK2 with or without an empty vector and 4 ng of pRL-TK (Promega) as an internal control using lipofectamine 2000 (Invitrogen). The luciferase reporter plasmid used was either TOPFlash (Promega, containing the wild-type TCF-binding site or FOPFlash (Millipore/Upstate) containing the mutated TCF-binding site (negative control). After 48 h, luciferase activities were measured in a luminescence plate reader (TECAN), and data were normalized for background Renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

**Western blotting**

Cells were lysed on ice for 30 min using RIPA buffer (Roche). Protein concentrations were determined using the Bradford method. For detection of altered β-catenin levels, the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) were used to extract cytoplasmic and nuclear protein, according to the protocol provided by the manufacturer. Proteins (30 μg per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). After blocking with 5% dry milk in TBST for 1 h at room temperature, the membranes were incubated with the primary antibody against human DKK2 (1:1000), β-catenin (1:1000), β-tubulin (1:2000), p-focal adhesion kinase (pAK, 1:1000), matrix metalloproteinase-2 (MMP-2; 1:1000), MMP-9 (1:500; Cell signalling), TBP (1:2000; BD Biosciences), mouse DKK2 (1:300; Abnova) and β-tubulin (1:1000; Abcam, Cambridge, UK) in dilution buffer overnight at 4°C. After washing three times with TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:3000) or goat antimouse (1:3000) antibodies combined with IgG (Cell signalling) for 1 h at room temperature. The expression of specific proteins was detected through the use of an electrochemiluminescence kit (Millipore) following the manufacturer’s instructions.

**Cell proliferation assay**

Cell proliferation was measured using the cell counting kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer’s instructions. Briefly, 10 μl/well of cell counting kit-8 reagent was added to 100 μl of medium containing cells in each well of a 96-well plate and left for 1 h under a humidified atmosphere 5% CO₂ at 37°C. For colorimetric analysis, the absorbance at 450 nm was recorded using a microplate reader (Multiskan MK3; Thermo Scientific). Each experiment was performed in triplicate.

**Cell migration assay**

Migration assay was performed using Boyden chambers containing polycarbonate filters (with an 8-μm pore size) (Millipore). Cells were harvested by trypsinization, resuspended in serum-free medium at a density of 5 × 10⁴ cells/well and placed in the upper compartment of the chamber in triplicate. Medium containing 20% fetal bovine serum was added to the lower chamber. After incubation at 37°C for 8 h, cells on the upper side of the membrane were removed using sterile cotton swabs. Cells adhering to the lower surface were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. Cells were counted using a light microscope (Nikon TE300, Tokyo, Japan) at ×200 magnification. Five random fields were selected for examination on each membrane, and results were expressed in terms of the migrated cells per field.

**Cell invasion assay**

Cell invasion assays were completed using 12-well BD Biocoat Matrigel invasion chambers (BD Biosciences). Cells were resuspended in serum-free medium at a density of 1 × 10⁵ cells/well in the upper chamber. Medium containing 20% fetal bovine serum was added to the lower chamber. After incubation at 37°C for 24 h, cells that had migrated through the membrane were fixed, stained and counted using a microscope (Nikon TE300). Five random fields were selected for examination on each membrane, and the results were expressed in terms of migrated cells per field. Each experiment was conducted in triplicate.

**Animal studies**

All animal experiments described in this study were performed according to the Guidelines for the Care and Use of Laboratory Animals provided by the Shanghai Jiao Tong University. Female nu/nu mice aged 4–6 wks were provided by the Chinese Academy of Sciences. Animal subjects were divided into groups of six mice per group and housed with free access to food and water. To verify the in vivo effect of DKK2 restoration on ovarian cancer cells, we did subcutaneous (s.c.) injections into both flanks with 1 × 10⁶ cells in 0.1 ml per site: on the left flank with pCMV6-DKK2 for SKOV3 and on the right flank with pCMV6 vector for SKOV3. To determine the effect of 5-aza-CAR-induced DKK2 gene expression and its ability to inhibit proliferation of ovarian tumor cells in nude mice, subjects were injected s.c. with SKOV3 cells. Thereafter, each subject received an intraperitoneal injection of 5-aza-CdR (0.25 mg/kg dissolved in phosphate-buffered saline (PBS) weekly for four consecutive weeks. Control group subjects were simultaneously administered equivalent volumes of PBS through intraperitoneal injection.
Subjects were monitored biweekly for evidence of tumor growth and killed when they became moribund or at 30 days after injection. Tumor volume was calculated from tumor dimensions collected at 3–4 days by caliper measurement of the shortest (A) and longest (B) tumor diameters. The formula $V = (A^2 \times B)/2$ was used to calculate tumor volume. Subjects were weighed and tumors were measured every 3–4 days. The CpG island methylation status of \textit{DKK2} promoter regions in xenograft tumors were analyzed by MSP, and the messenger RNA (mRNA) and protein expressions of \textit{DKK2} in xenograft tumors were analyzed by reverse transcriptase (RT)-PCR and western blotting.

Statistical analysis

All data were expressed as mean ± SD. Data analysis were carried out using Mann–Whitney U-tests, Kruskal–Wallis tests and Spearman rank correlation analyses. A $P$ value less than 0.05 ($P < 0.05$) was considered statistically significant. All calculations were performed using SAS 8.1 software (SAS Institute, USA).

Results

\textit{DKK2} expression in ovarian cancer cells and tumor specimens

\textit{DKK2} mRNA expression was analyzed by real-time RT-PCR in a set of ovarian tissue specimens, including cultured EOC cell lines (SKOV3 and ES-2 cells) and patient specimens of PEOC, benign ovarian tumor and normal ovarian tissue. The expression of \textit{DKK2} mRNA was barely detectable in SKOV3 and ES-2 cell lines. \textit{DKK2} mRNA levels in PEOC were significantly lower than levels observed in normal ovarian and benign tumors ($P < 0.05$). No significant differences were observed between the \textit{DKK2} mRNA levels of normal ovarian and benign tumor tissues ($P > 0.05$). Reduced \textit{DKK2} expression was observed with increasing FIGO (Federation International of Gynecology and Obstetrics) stage and histology, indicating that \textit{DKK2} expression is negatively correlated with FIGO stage and histology ($r = -0.777, P < 0.0001$, $r = 0.586, P = 0.0002$, respectively). Also, \textit{DKK2} expression was observed to be negatively correlated with ascite status ($r = -0.497, P = 0.0002$). No significant differences were observed based on patient age (<50 vs. >50; Table I). \textit{DKK2} protein expression was also demonstrated to be negatively correlated with tumor progression and tumor differentiation ($P < 0.0001$, $P = 0.0002$, respectively). Based on these findings, \textit{DKK2} warrants further investigation as a target of epigenetic silencing in PEOC.

![Fig. 1. Expression of DKK2 in human ovarian tissue samples and cell lines. (A) mRNA expression of DKK2 was analyzed by real-time RT-PCR. DKK2 mRNA levels in malignant ovarian tumor (M) were significantly lower than that in normal ovary (N) and benign tumors (B) (*$P < 0.05$) and were barely detectable in SKOV3 and ES-2 cell lines. (B) Representative immunohistochemical staining of DKK2 in human normal ovary (B1), benign ovarian tumor (B2) and ovarian carcinoma samples (B3). (C) Representative MSP and USP (unmethylated-specific PCR) of DKK2 in normal ovary (N), benign ovarian tumor (B), malignant ovarian tumor (M) and ovarian cancer lines (SKOV3 and ES-2). Positive control (PC), normal lymphocyte DNA treated with SssI methylease; negative control (NC), normal lymphocyte DNA; H2O, reaction without template DNA; M, methylation; U, unmethylation. J.Zhu et al.](#)
Table 1. Clinicopathological features of ovarian tissue with regard to the relative DKK2 expression and methylation

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N, number of total samples in group; n, number of samples from N; NS, not significant.

Analysis of DKK2 methylation in ovarian samples

DKK2 contains CpG islands in and around the transcription start site. MSP analysis revealed that complete methylation of DKK2 in SKOV3 and ES-2 ovarian cancer cell lines correlated with severely reduced or absent DKK2 expression. DKK2 promoter hypermethylation was observed in 27 of 50 (54%) of all ovarian cancer specimens, whereas DKK2 was unmethylated in all normal ovarian and benign tumor specimens examined (P < 0.05; Figure 1C). Additionally, DKK2 methylation frequencies were higher in relatively later stage (FIGO III-IV) specimens than early-stage carcinomas (FIGO I-II; P < 0.05). A significant difference in DKK2 methylation frequency was observed among histological types (G 1–G3; P < 0.05). This data revealed that DKK2 hypermethylation may be a neoplastic feature of ovarian carcinomas (Table I).

In vitro and in vivo suppression of ovarian cancer cell growth and invasion by DKK2

The effect of DKK2 on ovarian cancer cells was examined by restoring expression of DKK2 in SKOV3 and ES-2 cells in which endogenous expression had been silenced by DNA hypermethylation. This was accomplished using stable transfection with a DKK2 expression construct. Successful reestablishment of DKK2 expression in several individual clones (no. 1–3) was verified by real-time RT-PCR and western blotting (Figure 2A). Notably, no change in DKK2 expression was found in mock cells. To test whether DKK2 affects ovarian cancer cell growth and invasion, proliferation assays and Biocat Matrigel invasion assays were completed using stably transfected SKOV3 and ES-2 cells that over-expressed DKK2. As shown in Figure 3A, introduction of DKK2 significantly inhibited both SKOV3 and ES-2 cell proliferation. Additionally, in vitro migration and invasion assays using the Boyden Chamber System indicated a strong reduction in the migratory and invasive potential for cells in which DKK2 expression was reestablished compared with mock cells (Figure 3B and 3C).

We s.c. injected SKOV3 cells and DKK2-overexpressed SKOV3 cells in vivo in nude mice and found that mice inoculated with DKK2-infected SKOV3 cells developed tumors of an average volume of 1.47 ± 0.25 cm³, a volume significantly smaller than observed in untreated SKOV3 cell tumors (2.08 ± 0.46 cm³, P < 0.001; Figure 3D).

Tumor-suppressing effects of DKK2 through the canonical Wnt signal pathway

The effect of DKK2 on Wnt/β-catenin signaling in ovarian cancer cells was tested by assessment of the β-catenin status in SKOV3 and ES-2 cells, exhibitors of endogenous nuclear β-catenin expression. A dramatic decrease in β-catenin expression was observed in DKK2-transfected cells compared with mock cells (Figure 2A). Furthermore, luciferase reporter assay in SKOV3 and ES-2 cells showed that TCF activity was significantly suppressed in DKK2-transfected cells compared with mock cells (P < 0.001; Figure 2B). Thus, it is reasonable to assume that DKK2 functions as a novel component of the negative feedback loop that inhibits Wnt signaling.

Based on these findings, C-Myc and cyclin D1, the well-known target genes of the Wnt canonical pathway, are probably to play a role in ovarian tumor proliferation. Expressions of C-Myc and cyclin D1 were confirmed to be significantly downregulated in DKK2-transfected cells (Figure 4A). To investigate this mechanism in more detail, the expression of FAK, MMP-2 and MMP-9 in ovarian cancer cells was evaluated by western blotting, indicating significant downregulation of FAK and MMP-2 expression in DKK2 transfectants (Figure 4B). The expression of MMP-9, however, was not significantly altered by DKK2 transfection (data not shown).

In vivo and in vitro DKK2 gene restoration suppresses tumor growth by 5-aza-CdR treatment

The potent DNA methyltransferase inhibitor 5-aza-CdR can covalently bind to DNA methyltransferases, effectively inhibiting their catalytic activity and ultimately leading to demethylation. Treatment with 5-aza-CdR was conducted to determine the in vitro growth suppression in SKOV3 and ES-2 ovarian cancer cells by increased DKK2 expression. Treatment with 5-aza-CdR was observed to significantly inhibit SKOV3 and ES-2 cell proliferation by 60.4% and 55.4%, respectively, compared with the control (Figure 5A). Similarly, the treatment increased DKK2 mRNA levels in a dose-dependent manner (Figure 5B), and MSP analysis clearly indicated that treatment induced demethylation of a promoter region in the DKK2 gene (Figure 5C).

Subsequently, 5-aza-CdR treatment was examined for its restorative effect on DKK2 expression in vivo. On day 36 after tumor implantation, tumor volume and weight in the 5-aza-CdR treatment group were significantly reduced to 43.6% and 41.6%, respectively, compared with the control (Figure 5D). In addition, no mice died from treatment during the course of this experiment (data not shown). DKK2 mRNA and protein expression dramatically increased in xenograft tumor tissues from the 5-aza-CdR treatment group compared with the control (Figure 5E). Treatment with 5-aza-CdR was observed to suppress SKOV3 tumor growth in the xenograft model by restoring DKK2 expression. These findings suggest that treatment with 5-aza-CdR could be an effective therapy against human ovarian cancer. Furthermore, DKK2 may be a potential target for improved future ovarian cancer therapies.

Discussion

The DKK family, particularly DKK2, may play a crucial role in the development of new therapeutic treatments for ovarian cancer, the most lethal of all gynecological cancers. This new method of understanding cancer and potentially producing new treatment regimes can be attributed to the recent recognition of the role played by epigenetic
Fig. 2. (A) Expression of DKK2 and β-catenin in stable DKK2-transfected SKOV3 and ES-2 cells. 1: real-time PCR; 2: western blots; β-tubulin and TBP were used as a loading control. β-catenin expression was dramatically decreased in DKK2-transfected cells compared with mock cells. (B) Comparison of luciferase activity in mock and DKK2-transfected SKOV3 and ES-2 cells by using phRL-TK, TOP-FLASH (the wild-type TCF reporter) or FOP-FLASH (the mutant TCF reporter). The relative TCF activity was significantly suppressed in DKK2-transfected cells compared with mock cells (***P < 0.001).
mechanisms in the development and progression of ovarian cancer. Already, such epigenetic alterations have been applied in the development of novel biomarkers for diagnosis, prognosis, therapeutic prediction and monitoring of ovarian cancer (9,23–26). DNA methylation inhibitors were the first epigenetic drugs proposed for use as cancer therapeutics. 5-aza-CdR is effective in the treatment of myelodysplastic syndrome. It has antitumor effects in vitro and in vivo in different solid cancers, including ovarian cancer (27–30). Previous works from the ovarian cancer literature have demonstrated that demethylating agents can inhibit the growth of cell lines enhancing activity of carboplatin (31). Furthermore, treatment of xenografts with deacetylase (DAC) and the histone DAC inhibitor belinostat enhanced the activity of cisplatin, which is associated with the upregulation of human mutL homolog 1 (32). Increasing evidence suggests that the epigenetic silencing of the genes encoding Wnt antagonists plays an important role in the development and progression of many different

Fig. 3. DKK2 suppresses the growth and invasion of ovarian cancer cells in vitro and in vivo. Comparison of cell proliferation (A), migration (B) and invasion (C) in mock and DKK2-transfected SKOV3 and ES-2 cells. The cell proliferation, migratory and invasive potential of DKK2-transfected cells showed a strong reduction compared with mock controls (*P < 0.05; **P < 0.01). (D) The effects of DKK2 on tumorigenicity in nude mice. Mean tumor volume and weight in the DKK2-transfectant group were significantly reduced compared with the only vector group (**P < 0.01; *P < 0.05).
types of cancer (33–36), making better understanding of these mechanisms a necessity for improvement in cancer research and treatment.

The DKK family is composed of at least four members, DKK1–DKK4, that act as inhibitors of Wnt/β-catenin signaling. Several studies have reported that DKK1 is methylated in colorectal cancer and breast tumors (37,38). In addition, silencing of DKK3 function has been identified as an epigenetic promoter for methylation in gastric, breast cancer and lymphoblastic leukemia (9,39,40). Similarly, epigenetic silencing of DKK2 has also been reported in gastric cancer (7), malignant melanoma (22), renal cancer and renal cell carcinoma (21). Cumulatively, these findings suggest that members of the DKK family may be intrinsically linked with cancer cell proliferation.

No reports have previously indicated DKK2 expression in EOC tissues or its effect on Wnt/β-catenin signaling in EOC, as examined in

![Fig. 4.](image-url)
this study. Examination of the expression and methylation of DKK2 in cultured SKOV3 and ES-2 ovarian cancer cell lines as well as 78 variant patient tissues showed that DKK2 is highly downregulated in EOCs. DKK2 expression levels are the highest DKK2 tends to be present predominantly in its unmethylated form in normal and benign tumor tissues, whereas DKK2 is methylated in most ovarian carcinomas. Results of this study suggest that DKK2 is epigenetically silenced by methylation in ovarian cancer cells. Furthermore, increased methylation was shown to correlate with advanced ovarian carcinoma stage (FIGO III-IV), pathologic stage and ascites status. Notably, although aging has been shown previously to be an important risk factor for the development of neoplasia and increased methylation, current findings suggest that aging is not a contributor to DKK2 hypermethylation in ovarian cancer. This study provides the novel suggestion that DKK2 promoter hypermethylation occurs in ovarian carcinomas and plays a role in the development of these tumors.

Human DKKs (DKK1–4) have been found to be primary inhibitors of Wnt signaling. They act by binding to the transmembrane receptors Krm and LRP5/6, a corepressor of the Wnt/Fz receptor (41). In other forms of cancer, such as renal cell carcinoma, epigenetic studies have been completed showing no significant relationship between DKK2 methylation and β-catenin expression (21). The action of DKK2 in this study

Fig. 5. In vivo and in vitro DKK2 gene restoration suppresses tumor growth by 5-aza-CdR treatment. (A) Treatment with 5-aza significantly inhibited SKOV3 and ES-2 cell proliferation compared with the control treated with PBS (***P < 0.001). (B) DKK2 mRNA levels in SKOV3 and ES-2 cells were increased by 5-aza treatment in a dose-dependent manner. (C) Mass spectrometry-PCR analysis showed demethylation of DKK2 gene in SKOV3 cells after 5-aza treatment. C, SKOV3 cells treated with PBS; DAC, SKOV3 cells treated with 5-aza-CdR (DAC); positive control (PC), normal lymphocyte DNA treated with SssI methylase; negative control (NC), normal lymphocyte DNA; H2O, reaction without template DNA; M, methylation; U, unmethylation. (D) Mean tumor volume and weight in the 5-aza treatment mice group were significantly reduced compared with the control treated with PBS (***P < 0.001 *P < 0.05). Closed column: PBS treatment group; Open column: 5-aza treatment group. (E) DKK2 mRNA and protein expression in xenograft tumor tissues from the 5-aza treated group was drastically increased compared with that from control group (**P < 0.01).
could be explained by the previously observed abnormal activation of the Wnt/β-catenin signaling pathway associated specifically with ovarian carcinomas; however, the specific Wnt ligand and pertinent downstream mechanisms of this process have not been fully documented. Nevertheless, the expression of many Wnt target genes, including CCND1 (cyclin D1) (42), various MMPs (43,44) and MET (45) is observed in EOC. This study provides more detail on the prospective role of DKK2 in regulation of Wnt/β-catenin signaling, a mechanism that remains poorly understood and understudied in ovarian carcinogenesis.

**DKK1** has been reported to suppress cell growth and induce apoptotic cell death in both mesothelioma and HeLa cells (46,47). Several studies have indicated that DKK3 may function as a putative tumor suppressor in human cancer, in which it may be involved in apoptosis (9,48). In contrast, the function and biological role of DKK2 remains poorly understood. In this study, the relationship between DKK2 expression status and clinicopathological factors suggests that DKK2 may be involved in ovarian tumor growth and metastasis. This study uses the nude mouse model to show that tumor growth was reduced in vivo by overexpression of DKK2 in mice receiving s.c. injections or 5-aza-CdR treatment, responsible for reestablishment of DKK2 expression. In vitro analysis revealed that proliferation, migration and invasion of ovarian cancer cells decreased after DKK2 transfection, suggesting that DKK2 is a potential target for the treatment of human ovarian cancer.

The results of this study confirm previous findings reported in gastrointestinal cancer cells by Sato et al. (7). Furthermore, Fidler et al. reported that FAK and MMPs are associated with metastases (49), another result confirmed by this study. Secretion and activation of MMP-2 and MMP-9 may largely be responsible for the mechanism of decreased motility, invasiveness and metastasis of these cells (50). Increased FAK expression and activity frequently correlate with metastatic disease and poor prognosis (51). In accordance with these findings, current results show that DKK2-overexpression downregulates the expression of MMP-2 and FAK, thus inhibiting cell migration and invasion.

The Wnt signaling pathway has been studied thoroughly in variant forms of cancer, most notably colorectal cancer (52). Few previous studies have examined Wnt signaling in ovarian cancer, with these limited reports producing controversial results (53). In ovarian cancer cell lines examined, β-catenin status in SKOV3 and ES-2 cells both exhibited significantly reduced endogenous nuclear β-catenin expression upon reestablishment of DKK2 expression. Furthermore, cells transfected with DKK2 showed suppression of relative TCF activity. The downstream genes involved in Wnt signaling, C-Myc and Cyclin D1, were also downregulated significantly after reestablishment of DKK2 expression. Cumulatively, these results suggest that increased expression of DKK2 may suppress tumor phenotypes through the Wnt canonical pathway.

A novel demonstration of the relationship between epigenetic silencing of the putative Wnt inhibitor DKK2 and tumor growth, migration and invasion in ovarian cancer was produced in this study. Promoter hypermethylation of DKK2 may have additional applications as a biomarker for ovarian cancer screening. Treatment with 5-aza-CdR suppressed the growth of SKOV3 tumors in a xenograft model by restoring DKK2 expression, suggesting that DKK2 may also be a potential target for the treatment of ovarian carcinoma. The notable development of Wnt signaling inhibitors may lead to new ovarian cancer treatments, though extensive further investigations will be required to research and document the mechanisms leading to the inactivation of DKK2.

**Supplementary material**

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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**References**


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