Original Research

DNA methyltransferase inhibitor-mediated apoptosis in the Wnt/β-catenin signal pathway in a renal cell carcinoma cell line

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Abstract

The Wnt signaling pathway is activated in most cancer types when Wnt antagonist genes are inactivated. Glycogen synthase kinase 3 (*GSK3β*) is an important regulator of the Wnt/β-catenin signaling pathway. The mechanisms underlying *GSK3β* regulation of neoplastic transformation and tumor development are unclear. Studies have raised the possibility that the Wnt signaling pathway may be implicated in renal cell carcinoma (RCC). Therefore, in the present study, we hypothesize that the expression and methylation status of the secreted frizzled-related protein 2 (*sFRP2*) gene, one of the secreted antagonists that bind Wnt protein, and re-expression of this gene with the demethylation agent (5-aza-2′-deoxycytidine; DAC) may induce apoptosis in RCC cells. To test this hypothesis, we investigated the relationship among epigenetic inactivation of *sFRP2* and *p-GSK3β* (Ser9) and other Wnt antagonists (*sFRP1*, *DKK3*, *WIF-1*) and apoptotic factors (*Bax* and *Caspase3*) as well as the anti-apoptotic factor *BCL2*. Our results indicate that DAC-mediated inhibition of DNA methylation led to a re-activation of *sFRP2* expression and increased expression levels of the Wnt antagonists and apoptotic factors. In contrast, the level of β-catenin (CTNNB1) expression decreased. The *p-GSK3β* (Ser9) protein level in Caki-2 cells was significantly down-regulated, while the DNA fragmentation rate increased after treatment with 5 μM DAC at 96 h. Our data show that *sFRP2* functions as a tumor suppressor gene in RCC and that its restoration may offer a new therapeutic approach for the treatment of RCC. Moreover, our study draws attention to the regulatory features of epigenetic molecules and analyses their underlying molecular mechanisms of action and their potential use in clinical practice.

Keywords: Wnt antagonist, β-catenin, DNA methylation, RCC, *sFRP2*, *GSK3β*, apoptosis

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Introduction

Epigenetic mechanisms play a crucial role in the regulation of gene expression, and different epigenetic processes are involved in gene silencing. The silencing of cancer-associated genes by the hypermethylation of CpG islands within the promoter and/or 5′ regions is a common characteristic of human cancer cells and is often associated with a partial or complete transcriptional block.[1] Altered DNA methylation patterns have been widely reported to be a critical factor in both the development and progression of cancer.

Wnt/β-catenin signaling includes β-catenin-dependent (canonical) and β-catenin-independent (non-canonical) pathways. The canonical Wnt signaling pathway activates signal transduction pathways to control a wide variety of cellular processes such as the determination of cell fate, proliferation, migration and polarity. Signaling is initiated by the binding of Wnt ligands to the secreted frizzled-related (*sFRPs*) family of proteins and receptors and the low-density lipoprotein (LDL) receptor-related protein 5 and 6 (LRP5/LRP6) co-receptors.[2] Constitutive activation of the Wnt signaling pathway as a result of epigenetic alterations in Wnt antagonists (*sFRPs*, *DKK3* and *WIF-1*) provokes the accumulation of nuclear β-catenin and results in a functional transcription factor complex and the expression of downstream target genes. When a Wnt signal is absent, glycogen synthase kinase (*GSK3β*), whose activity is regulated by site-specific phosphorylation, promotes the proteasomal degradation of β-catenin. The full activity of *GSK3β* generally requires phosphorylation at tyrosine 216 (Tyr216), and conversely phosphorylation at serine 9 (Ser9) inhibits *GSK3β* activity (Figure 1(a)). More than 40 proteins are substrates of *GSK3β*, which is an important regulator of the Wnt/β-catenin signaling pathway. One of the most well-known substrates of *GSK3β* is β-catenin. When Wnt binds to frizzled-related protein (Frz), its receptor dishevelled

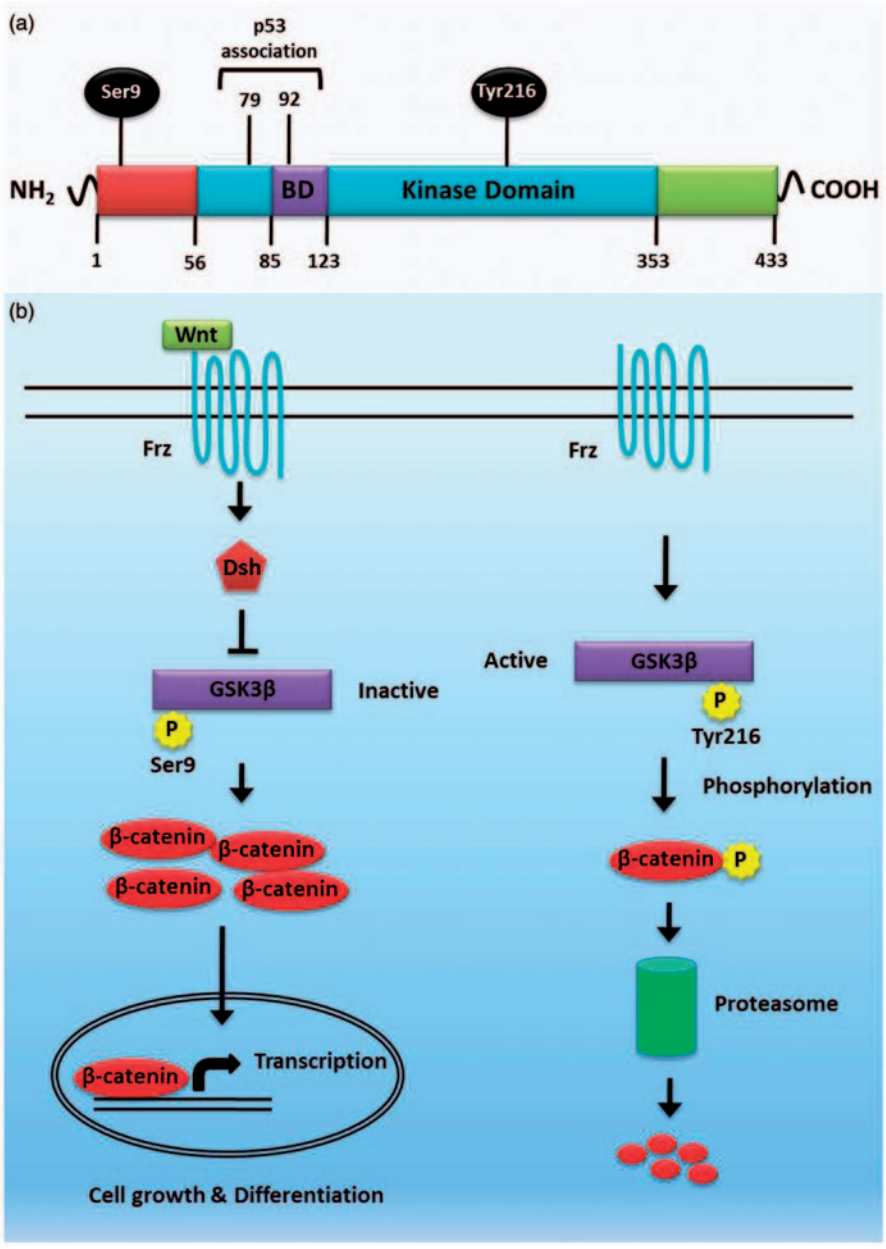

Figure 1 GSK3β structure and role of GSK3β in the Wnt pathway. (a) GSK3β is a 46-47 kDa protein consisting of 433 amino acids in humans. The protein contains a N-terminal domain, a kinase domain and a C-terminal domain. Phosphorylation of Tyr216 located in the T-loop (activation site) facilitates substrate phosphorylation by GSK3β. Phosphorylation of GSK3β at Ser9 in N-terminal region leads to inhibition of its kinase activity. Binding domain (BD) includes GSK3β-specific binding sites for substrates and protein complexes (e.g. p53). (b) Phosphorylation and activation of GSK3β. GSK3β protein itself undergoes phosphorylation events; Tyr216 phosphorylation leads to its activation and Ser9 phosphorylation results in inhibition of its activity. Note that there are other regulatory mechanisms of each pathway; this figure provides a basic overview for simplicity. (A color version of this figure is available in the online journal)

(Dsh) is recruited to the cell membrane. *GSK3β* is inhibited by the activation of Dsh. Consequently, β-catenin accumulates in the cytoplasm and is subsequently translocated into the nucleus to switch on the transcription of specific target genes, leading them to act as oncoproteins (Figure 1(b)).

Recent studies indicate that secreted Wnt antagonists have important roles in the pathogenesis of renal cell carcinoma (RCC).[3–9] Their results have been supported by experimental evidence showing that the functional loss of Wnt antagonists, for example by promoter hypermethylation, can contribute to constitutive activation of the Wnt pathway which results in carcinogenesis through dysregulation of cell proliferation and differentiation. Several Wnt signaling components have been examined in RCC. While studies suggest that Wnt signaling is constitutively active in RCC, the molecular mechanisms differ considerably from those in other human carcinomas. The methylation frequency of the Wnt antagonist genes could serve as an excellent epigenetic biomarker panel for the detection, staging and prognosis of RCC.[9] However, the functional

role of constitutive activation of the Wnt/β-catenin signal pathway involved in the pathogenesis of RCC still remains unclear. A DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (DAC), has been used to reverse methylation and reactivate the expression of silenced genes. DAC could suppress the growth of various tumors *in vitro*, and showed clinical utility against hematopoietic malignancies.[10] It has been reported that the expression levels of several Wnt antagonists were downregulated epigenetically in renal cancer.[4,11]

The purpose of the present study is to investigate the silencing mechanism of the Wnt antagonist *sFRP2* and the effects of the phosphorylation of *GSK3β* by DAC on the Wnt/β-catenin signaling pathway in the human renal carcinoma cell line Caki-2. We hypothesized that *sFRP2* is densely methylated in its promoter region, and functions as a tumor suppressor gene in RCC. To address this issue, we checked the expression levels of *sFRP1*, *sFRP2*, *DKK3*, *WIF-1*, *CTNNB1*, *BCL-2* and *Bax* genes in RCC cells. Inhibition of the Wnt/β-catenin signaling pathway may offer a novel strategy for RCC treatment. As far as we are concerned, a wide-ranging research tackling this relationship has not been undertaken to date.

Materials and methods

Cell culture conditions and cell proliferation assay

The human renal cancer cell line Caki-2 (purchased from the American Type Culture Collection, ATCC, Manassas, VA, USA) was maintained in RPMI-1640 medium with L-glutamine, 10% fetal bovine serum and 100 U/mL penicillin-100 mg/mL streptomycin (Sigma-Aldrich) in 5% CO_2:95% air at 37°C. DAC was obtained from Sigma (St. Louis, MO, USA).

A total of 5×10^3 cells per well were seeded in 200 μL of complete culture medium containing various concentrations of DAC (ranging from 5 μM to 10 μM), incubated for 24, 48, 72 and 96 h to determine its cytotoxic and apoptotic effects. Cells treated with 0.1% dimethylsulfoxide (DMSO) served as a solvent control. Each DAC concentration was repeatedly incubated in four wells for each incubation period to find the most efficient dose(s) and incubation period(s). IC_{50} values defined the concentrations of the chemical agents causing a 50% decrease in cell viability. We measured the cytotoxic activity using the WST-1 assay (Roche, Germany), following the manufacturer's instructions as previously described by Fortmüller *et al.*[12] The spectrophotometrical absorbance of the samples was measured at 450 nm in an enzyme-linked immunosorbent assay (ELISA) microplate reader (Spectramax M3, Molecular Devices, USA). Cell proliferation was determined by colorimetric ELISA with 5-bromo-2-deoksiuridin (BrdU) using commercial cell proliferation ELISA (Roche, Germany). The cells (5×10^3) were seeded in 96 well plates and then incubated for specified doses and periods. The medium was then removed and the cells were incubated for 2 h at 37°C with a BrdU-labeling solution containing 10 μM BrdU. The assay was performed according to the manufacturer's instructions. Absorbance was measured at 450 nm in an ELISA microplate reader (Spectramax M3, Molecular Devices, USA). A four-day treatment with 5 μM of DAC showed the maximal effect on one of the WNT signals (*SFRP2*). Therefore, in this study we chose 5 μM of DAC for four days as the treatment. Each treatment group had three replicates.

Methylation-specific PCR (MSP) and DNA and RNA isolation

To determine an epigenetic alteration of secreted frizzled-related protein 2 (*SFRP2*) gene, Caki-2 cells (1×10^6) were seeded in six well plates and were treated with 5 μM DAC for 24, 48, 72, and 96 h. The culture medium and DAC were refreshed every 24 h for four days. The total RNA and genomic DNA were isolated before and after DAC treatment by using methylation specific polymerase chain reaction (MSP) and real-time PCR. The methylation status in *SFRP2* was determined by chemical treatment with sodium bisulfite and subsequent MSP analysis. Bisulfide modification of genomic DNA was performed by using an EZ DNA methylation kit (Zymo Research). Bisulfite-treated genomic DNA was amplified using either a methylation-specific or an unmethylation-specific primer set. The primer sequences were 5′-GGG TCG GAG TTT TTC GGA GTT GCG C-3′ (sense) and 5′-CCG CTC TCT TCG CTA AAT ACG ACT CG-3′ (antisense) for the methylated-reaction and 5′- TTT TGG GTT GGA GTT TTT GGA GT TGT GT-3′ (sense) and 5′-AAC CCA CTC TCT TCA CTA AAT ACA ACT CA-3′ (antisense) for the unmethylated reaction. The MSP conditions were as follows: denaturation at 95°C for 5 min, 35 cycles of amplification (95°C, 30 s; 60°C, 30 s and 72°C, 30 s) and a final elongation step at 72°C for 10 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

After treatment with specified DAC concentrations for specific periods, genomic DNA was extracted using a High Pure PCR Template Preparation kit (Roche, Germany) and total RNA was extracted using a High Pure RNA Isolation kit (Roche, Germany). DNA and RNA specimens were stored in at −20°C and −80°C, respectively. The DNA and RNA concentrations were determined using a NanoDrop (ND-1000, Montchanin, DE, USA) spectrophotometer.

Cell death detection ELISA and caspase-3 (CASP3) protein activity assay

The induction of apoptosis was determined by using the Cell Death Detection ELISA Plus kit (Roche, IN, USA). The assay is based on quantitative immunoassay principles using a monoclonal antibody directed against DNA and histones. After incubation with various drug concentrations for desired periods, the 96 well plates were centrifuged (200 g) for 10 min. The supernatant was discharged, lysis buffer was added and the samples were incubated at room temperature for 30 min in accordance with the manufacturer's protocol. Anti-histone biotin and anti-DNA peroxidase antibodies were added to each well and the mixture was incubated at room temperature for 2 h. After three washes, the peroxidase substrate was added to each well and the plates were read at 405 nm.

The levels of CASP3 activity in Caki-2 cells were assayed by using a luminescent assay according to the manufacturer's experimental instructions (PathScan Cleaved Caspase-3 [Asp175] Sandwich ELISA; Cell Signaling Technology, Danvers, MA, USA). The purpose was to detect the pro-apoptotic effects of different doses of DAC at specified incubation periods, given that this family of protease plays a key effector role in apoptosis in mammalian cells. Adding the reagent to the wells resulted in cell lysis. This was followed by Caspase cleavage of the substrate and generation of a luminescent signal produced by luciferase, which was proportional to the amount of Caspase activity present. CASP3 activity was analyzed by reading the absorbance at 450 nm.

Quantitative real-time PCR assays

Total RNA (1 μg) was reverse-transcribed in a 20 μl reaction mixture using random hexamers and a high fidelity cDNA transcript synthesis kit (Roche, Germany) according to the manufacturer's instructions. *SFRP1, SFRP2*, *DKK3*, *WIF-1, CTNNB1*, *BCL-2* and *Bax* mRNA expression levels were measured by using real-time PCR. Probes and primer sets for each gene were designed at the Probe Finger Design Assay Center website. The primers and probe numbers are listed in Table 1. The 10 μl reaction mixture in all 96 well plates contained 1 × LightCycler Probe Master mix, 2.5 pmol of each primer, 1 pmol of UPL probe, 4 mM $MgCl_2$ and 1 μM cDNA. All PCR reactions were performed in the LightCycler 480 instrument (Roche Diagnostics). To normalize the results obtained by quantitative reverse transcriptase PCR, we analyzed the expression of the *GAPDH* housekeeping gene. Each sample was tested in triplicate. The PCR efficiency for each gene was tested by serial dilutions of all genes. The amplification efficiencies of the target genes and those of *GAPDH* were approximately equal.

Protein isolation and phospho-GSK3β (Ser9) protein western blotting

Lysates containing 100 μg of protein were mixed with a loading buffer with 5% β-mercaptoethanol and heated for 5 min at 100°C. The protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes by semi-dry blotting. SDS–PAGE was performed via standard procedures according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Membranes were incubated in blocking buffer (tris-buffered saline [TBS], 0.1% Tween 20, and 5% low-fat dry milk) for 1 h at room temperature, followed by hybridization with anti-*p-GSK3β* (Ser9) antibody (Cell Signaling Technology, 1:1000 dilution), anti-*GSK3α/β* antibody (Cell Signaling Technology, 1:1000 dilution) and anti-β-actin antibody (internal control) (Santa Cruz Biotechnology, USA) at 4°C overnight. After three washes in TBS/0.1% Tween 20, the membranes underwent hybridization with a horseradish peroxidase-conjugated secondary rabbit IgG antibody (Santa Cruz Biotechnology, 1:5000 dilution) for 1 h at room temperature. After washing extensively in TBS/0.1% Tween 20, signals were detected by ECL (electrogenerated chemiluminescence) techniques using SuperSignal WestPico Chemilumiscent Substrate (Thermo, USA).

Statistical analysis

Each test point was measured in quadruplicate in three individual experiments. Statistical significance levels of differences in mRNA expressions were analyzed by using the pairwise fixed reallocation randomization test. The REST software tool (2009 version 2.013) was used for group-wise comparison and statistical analysis of relative expression results. Cell viability, alterations in apoptosis and necrosis were analyzed by using the one-way analysis of variance. Multiple comparison analysis was performed by using SPSS software (version 15.0, SPSS, Inc.). A p value less than 0.01 was considered to represent a statistically significant difference. The results were expressed in form of mean ± standard deviation (SD).

Results

Cytotoxic activity analysis of DAC on cell viability and proliferation

The Caki-2 human renal cancer cell line was exposed to different concentrations of DAC (5–10 μM) (Figure 2). Percentages of cell viability were found to be 53.27%, 52.54% and 51.62% for 5, 7.5 and 10 μM concentrations of DAC, respectively (Figure 2(a)). The figure only shows the results at 96 h but not 24 h, 48 h and 72 h due to the removal of methylation at 96 h. The IC_{50} values were verified by a cell proliferation ELISA assay when the cells in culture were treated with the same doses of DAC for all incubation periods (Figure 2(b)). The effective dose of DAC was found to be 5 μM for 96 h. There were no statistically significant

Table 1 Gene-specific primer sequences and probe numbers

Gene	Sense (5′ → 3′)	Antisense (5′ → 3′)	UPL Prob (#)
SFRP1	GCT GGA GCA CGA GAC	TGG CAG TTC TTG TTG AGC A	40
SFRP2	GCT AGC AGC GAC CAC CTC	TTT TTG CAG GCT TCA CAT ACC	83
DKK-3	TGG GAG GGG AAG AGA TTT AGA	GCA CAC ACC TGG GGA AAT AA	16
WIF-1	CCA GGG AGA CCT CTG TTC AA	TTG GGT TCA TGG CAG GTT	76
CTNNB1	TGG CCA TCT TTA AGT CTG GAG	CAA CAC AGA ATC CAC TGG TGA	37
BCL-2	GCA CCT GCA CAC CTG GAT	AGG CCA AAC TGA GCA GA	2
BAX	ATG TTT TCT GAC GGC AAC TTC	ATC AGT TCC GGC ACC TTG	57

differences among the cell viability and proliferation percentages when the cells were treated with 5 μM DAC and higher doses including the highest concentration (10 μM).

Methylation status of the sFRP2 promoter region and restoration of sFRP2 mRNA expression after DAC treatment

The methylation status of the *sFRP2* CpG islands was characterized in the cell line (Figure 3) to determine whether the loss of *sFRP2* expression resulted from promoter hypermethylation. An optimal dose of DAC was determined by MSP in Caki-2 human renal cancer cells to remove methylation. The results of MSP analysis were also confirmed by quantitative real-time PCR (Figure 3(a)). The Caki-2 cell line that indicated a low-level *sFRP2* gene expression displayed dense methylation in the *sFRP*2 promoter. After treatment with 5 μM DAC for 96 h, we found that expression levels of unmethylated *sFRP2* and its mRNA increased significantly. Treatment with 5 μM DAC for 96 h was sufficient for re-expression of the *sFRP*2 gene (Figure 3(b)).

Promotion of apoptosis

The results were evaluated with DNA fragmentation as a colorimetric. We found that DNA fragmentation rates increased after DAC treatments, particularly at 96 h (about 4.38 folds) compared with the control (Figure 4(a)). Also, the results were evaluated with cleaved Caspase3 levels through ELISA. There were dose-dependent increases (2.2 folds) at active *CASP3* protein level after treatment with DAC (5 μM) for 96 h ($P < 0.01$) (Figure 4(b)). The results were confirmed by the levels of *CASP3* mRNA expression. Similar results were observed for levels of *CASP3* mRNA, particularly after treatment with 5 μM DAC for 96 h which was found to be significant statistically ($P < 0.01$) (data not shown). These results suggested that the inhibition of cell viability decreased as a result of apoptotic cell death after DAC treatment.

The expression levels of sFRP1, sFRP2, DKK3, WIF-1, CTNNB1, BCL-2 and Bax mRNA regulated by DAC

Treatment with 5 μM DAC for 96 h resulted in an increase in *sFRP1* (2.52 folds), *sFRP2* (4.79 folds), *DKK3* (2.1 folds), *WIF-1* (2.02 folds) and *Bax* (1.8 folds) at 96 h (Figure 5(a)). On the other hand, treatment with the same dose of DAC for the same period reduced the level of *CTNNB1* by half. Restoration of *sFRP2* expression through the inhibition of

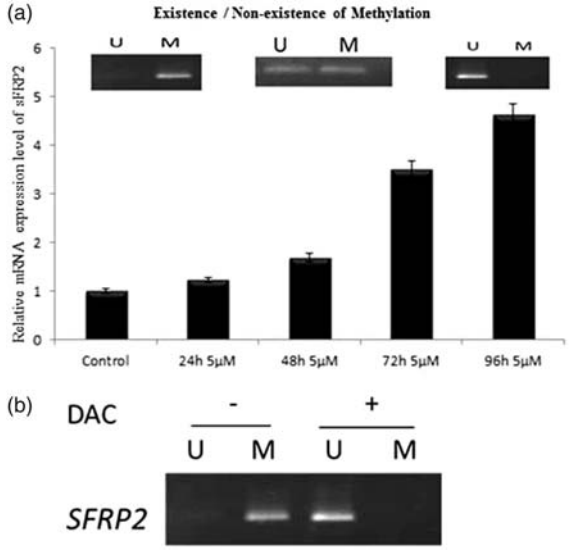

Figure 3 Levels of *sFRP2* expression in tumor cells analyzed by Methylation-specific PCR and mRNA expression quantified by real time PCR to observe whether methylation exists after 5 μM DAC treatment for different periods. U: Unmethylated; M: Methylated. (a) Methylation-specific PCR analysis of *sFRP2* in Caki-2 cells treated with 5 μM DAC for 24 h, 48 h, 72 h and 96 h. For 5 μM DAC, there is methylation at 24 h but methylation dwindles as treatment period increases and the process ends up with unmethylation at 96 h. As for the insets showing the existence/non-existence of methylation, note that the first inset on the left corresponds to the control group and 5 μM DAC for 24 h; second one in the middle corresponds to 5 μM DAC for 48 h and 72 h; the third one on the right corresponds to 5 μM DAC for 96 h. (b) Re-expression of *sFRP2* following treatment with 5 μM DAC for 96 h in renal cancer cells. In absence of DAC there exists methylation whereas inclusion of DAC causes unmethylation. Data points represent mean ± SD of triplicate experiments

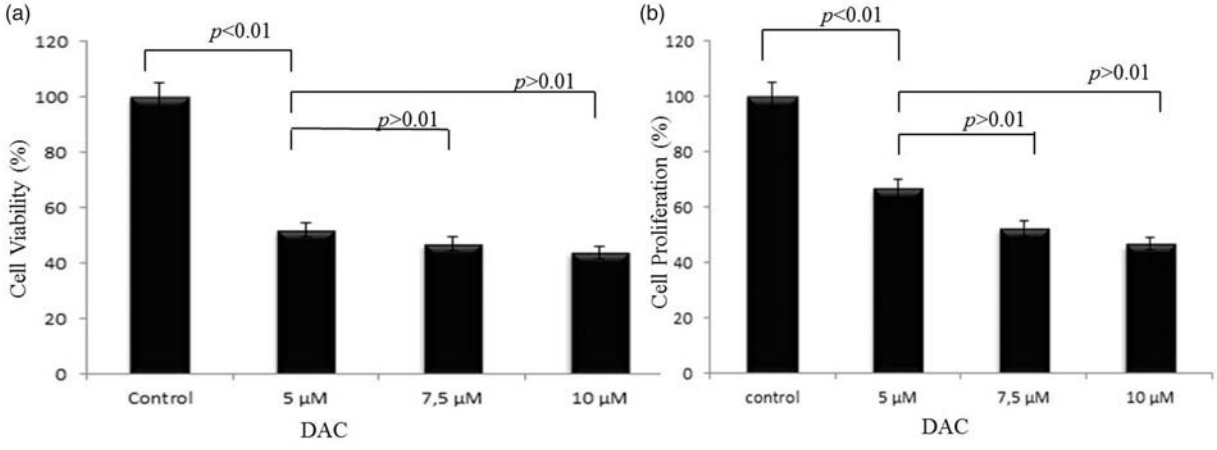

Figure 2 Graphical representation of WST-1 and ELISA assays. % change in (a) cell viability and (b) cell proliferation in Caki-2 cells treated with DAC (5–10 μM) for 96 h. Data points represent the mean ± SD of triplicate experiments

DNA methylation was inversely related to p-GSK3 the expression of *CTNNB1* and its target genes, including *BCL-2* mRNA transcript levels, with respect to the control in RCC cells (Figure 5(a)). The results for these genes were in agreement with the apoptosis and cell proliferation inhibition analysis. Also, we showed that anti-apoptotic gene *BCL-2* was down-regulated, although this is not a statistically significant result. As a result, DAC inhibited cell proliferation by down-regulating *CTNNB1* and induced apoptosis via decreased anti-apoptotic gene expression.

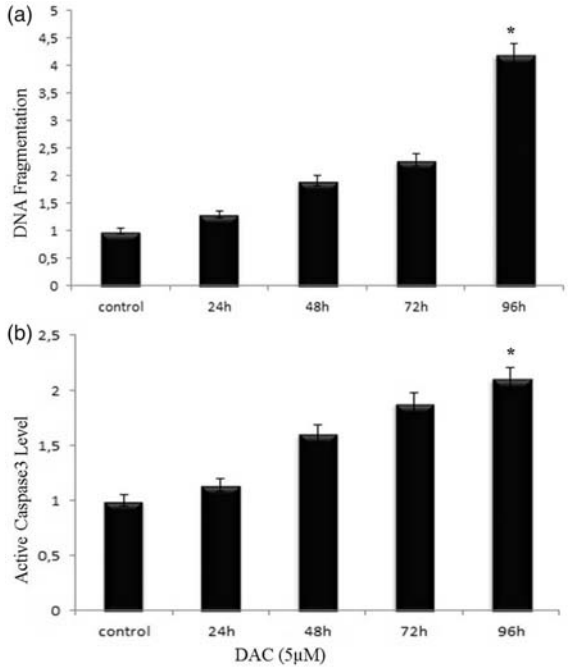

Figure 4 (a) DNA fragmentation in Caki-2 cells for 5 μM DAC treatment between 24 and 96 h. (b) Active *CASP3* protein levels for 5 μM DAC treatment between 24 and 96 h. Data points represent mean ± SD of triplicate experiments. *$P < 0.01$

Treatment with DAC inhibits the level of p-GSK3β (Ser9) protein

To determine the level of *p-GSK3β* (Ser9) (*GSK3β* inactivation marker) in human Caki-2 cells after DAC treatment, we exogenously added DAC to inhibit the phosphorylation of *GSK3β* at Ser9. Amongst the doses studied, the lowest level of *p-GSK3β* (Ser9) was observed for 5 μM DAC treatment (Figure 5(b)). The IC_{50} value of DAC was consistent with the cell proliferation levels detected earlier by using the WST-1 assay. Western blot analysis revealed that *p-GSK3β* (Ser9) protein levels in Caki-2 cells were significantly down-regulated after treatment with 5 μM DAC for 96 h in comparison to untreated cells. Protein levels of *GSK3β* remained unchanged in response to treatment with inhibitors.

Discussion

The Wnt/β-catenin signaling pathway is activated in many cancer types, and expression of Wnt antagonists is frequently epigenetically down-regulated.[13] Wnt signaling is likely to play a central role during the development of renal carcinoma. The function of several Wnt antagonists in RCC has been reported in several studies.[4–9,11,14,15] Despite the apparent importance of Wnt antagonists in cell proliferation, metastasis and survival in RCC, their potential molecular mechanisms involving RCC tumorigenesis have not been entirely characterized. We sought to determine whether DAC was effective in inhibiting the methylation status of the *sFRP2* promoter region and reactivating of

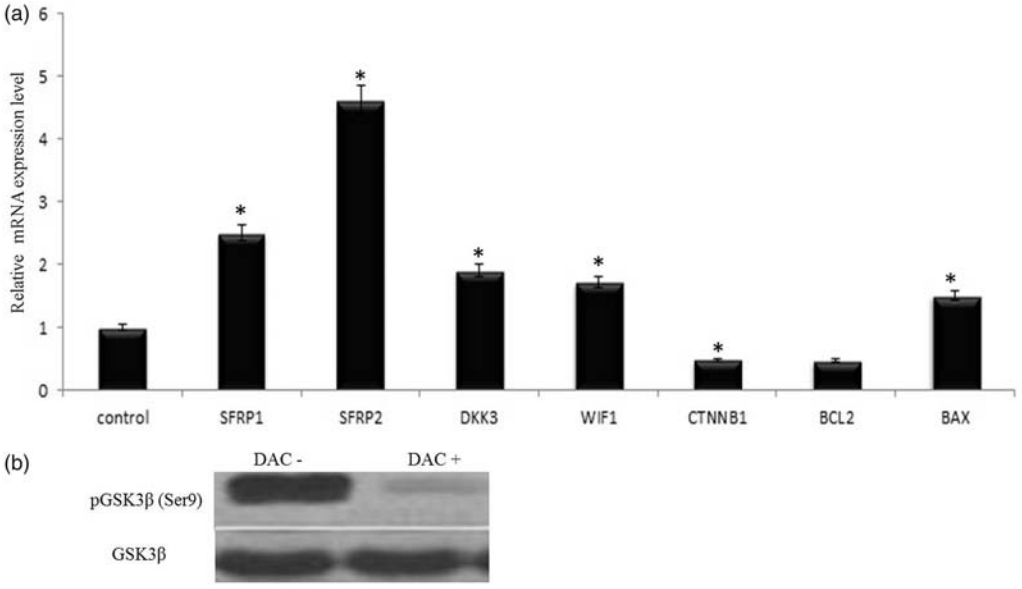

Figure 5 (a) Relative mRNA levels of *sFRP1*, *sFRP2*, *DKK3*, *WIF-1*, *CTNNB1*, *BCL-2* and *Bax* after treatment with 5 μM DAC for 96 h. The stars indicate that values are significantly different from the controls. Data points represent mean ± SD of triplicate experiments. *$P < 0.01$. (b) Representative Western blotting shows *pGSK-3β* (Ser9) and *GSK-3β* at 96 h after treatment with 5 μM DAC. The protein level of *pGSK-3β* (Ser9) fizzled out in the protein lysates extracted from the DAC-treated Caki-2 cells

the *sFRP2* expression in Caki-2 cells. This effect could modulate the expression of some other well-known Wnt antagonists, β-catenin and apoptotic or anti-apoptotic genes.

We found that some of the CpG sites of the *sFRP2* promoter were methylated in Caki-2 cells. A lower degree of methylation was found at 72 h, indicating that Caki-2 cells remain unmethylated with increased durations of exposure to DAC. Expression levels of *sFRP2* mRNA were significantly restored in RCC cells after treatment with 5 μM DAC for 96 h. Our result shows that the expression of the *sFRP2* gene silenced by methylation could be restored by an inhibitor of DNA methyltransferases (DNMTs). Such down-regulation of methylation status of the *sFRP2* promoter region was paralleled by an evident increase of *sFRP2* mRNA expression levels. There are no reports concerning *sFRP2* gene DNA methylation in Caki-2 cells except the report by Kawamoto *et al.*,[11] the results of which are consistent with ours. Furthermore, to our knowledge, there have been no previous reports showing the correlation between unmethylation in the *sFRP2* gene and down-regulation in the protein levels of the *p-GSK3β* (Ser9) gene in DAC-treated RCC cells. We showed that the methylation status of *sFRP2* is abolished by DAC and that this effect was related to the ability to restore *sFRP2* expression to decrease the protein level of phospho-*GSK3β*.

β-catenin has two cellular functions; (i) cell-cell adhesion and (ii) switch on/off canonical Wnt pathway. β-catenin becomes stabilized in the cytoplasm and subsequently translocates into the nucleus where it activates the expression of specific target genes such as *c-myc*, *cyclin D1*, *c-jun*, *survivin* and *MMP7*.[16–18] Steinhusen *et al.*[16] demonstrated that β-catenin was proteolytically cleaved by Caspase 3 during apoptosis. β-catenin was cleaved in N- and C-terminal regions at multiple positions but the central core region was not affected. As a result of this cleavage, β-catenin lost its own trans-activation potential. In our study, we observed that decreased β-catenin mRNA levels correlated with increased levels of active Caspase 3 protein and DNA fragmentation.

We further observed that the restoration of *sFRP2* expression through inhibition of DNA methylation was inversely related to transcript levels of *CTNNB1* and *BCL2* mRNA with respect to the control. However, after DAC treatment, *sFRP1*, *DKK3*, *WIF-1* and *Bax* mRNA were up-regulated in the Caki-2 cell line. In addition, such up-regulation was paralleled by an evident decrease in the expression level of *p-GSK3β* protein. We reached similar results for levels of the active Caspase 3 protein, revealing that DAC caused an increase in levels of the *CASP3* protein. This could be attributed to activation of apoptotic signal pathway. To validate our result, we also analyzed DNA fragmentation rates. The rates increased after DAC treatments, particularly with 5 μM for 96 h (about 4.38 folds) compared with the control. These results provide evidence that the mechanisms for inhibition of renal cancer by *sFRP2* are associated with its inhibition by β-catenin-dependent transcription of cell cycle-related Wnt target genes.

Hypermethylation of Wnt-antagonist genes plays an important role in the pathogenesis of renal tumors. Our data support the role for *sFRP2* as a tumor suppressor in RCC. Perhaps the loss of *sFRP2* is an early and aberrant molecular event in renal cell carcinogenesis. Since preclinical results have been promising, additional animal model and tissue sample studies need to be undertaken to determine whether over-expression of Wnt/β-catenin pathway is ''driving'' renal cell carcinogenesis. A larger series of RCC cell lines will need to be studied to confirm whether or not DNA methylation associated with *sFRP2* repression is a relevant mechanism in renal cell carcinoma. The identification of differential DNA methylation patterns in cancer cells may shed light on the processes underlying tumorigenesis.

Conclusion

Our study suggests that the use of DAC suppresses the canonical Wnt/β-catenin pathway and induces apoptosis in RCC cells. Also, *sFRP2* may have tumor supressor functions in renal cancer. In conclusion, the regulation of the Wnt/β-Catenin signaling pathway has the potential to provide effective strategies for the treatment of renal cancers.

Author contributions: Study design: EK and SS. Performed experiments: EK, NV and AY. Analysed output data: EK, SM, and SS. Manuscript preparation: EK, NV and AY.

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