Molecules and Cells

Concurrent Hypermethylation of *SFRP2* and *DKK2* Activates the Wnt/β-Catenin Pathway and Is Associated with Poor Prognosis in Patients with Gastric Cancer

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http://dx.doi.org/10.14348/molcells.2017.2245
www.molcells.org

Aberrant hypermethylation of Wnt antagonists has been observed in gastric cancer. A number of studies have focused on the hypermethylation of a single Wnt antagonist and its role in regulating the activation of signaling. However, how the Wnt antagonists interacted to regulate the signaling pathway has not been reported. In the present study, we systematically investigated the methylation of some Wnt antagonist genes (*SFRP2*, *SFRP4*, *SFRP5*, *DKK1*, *DKK2*, and *APC*) and their regulatory role in carcinogenesis. We found that aberrant promoter methylation of *SFRP2*, *SFRP4*, *DKK1*, and *DKK2* was significantly increased in gastric cancer. Moreover, concurrent hypermethylation of *SFRP2* and *DKK2* was observed in gastric cancer and this was significantly associated with increased expression of β-catenin, indicating that the joint inactivation of these two genes promoted the activation of the Wnt signaling pathway. Further analysis using a multivariate Cox proportional hazards model showed that *DKK2* methylation was an independent prognostic factor for poor overall survival, and the predictive value was markedly enhanced when the combined methylation status of *SFRP2* and *DKK2* was considered. In addition, the methylation level of *SFRP4* and *DKK2* was correlated with the patient's age and tumor differentiation, respectively. In conclusion, epigenetic silencing of Wnt antagonists was associated with gastric carcinogenesis, and concurrent hypermethylation of *SFRP2* and *DKK2* could be a potential marker for a prognosis of poor overall survival.

Keywords: β-catenin, DNA methylation, gastric cancer, prognosis, Wnt antagonists

INTRODUCTION

Gastric cancer (GC) is the fifth most commonly diagnosed cancer and the second primary cause of death worldwide (Ferlay et al., 2010). However, the etiology and pathogenesis of GC remain unclear. Both genetic and epigenetic factors play key roles in the development and progression of GC (Yoda et al., 2015).

Wnt/β-catenin signaling is known to regulate cell differentiation, proliferation, migration, and organogenesis during embryonic development (Cadigan and Nusse, 1997). Recent studies have revealed that aberrant activation of Wnt signaling is also involved in gastric carcinogenesis and progression

Received 18 October, 2016; revised 15 December, 2016; accepted 20 December, 2016; published online 26 January, 2017

eISSN: 0219-1032

(Ooi et al., 2009). Wnt ligands bind to the Frizzled (FZD) family of receptors and the LRP5/LRP6 coreceptor, subsequently activating the canonical and non-canonical Wnt pathways (Gonzalez-Sancho et al., 2004; Liu et al., 2005; Oishi et al., 2003). In the canonical pathway, signal transduction activates the protein Dishevelled (Dsh), which inhibits the activity of GSK3β, resulting in the accumulation of β-catenin in the cytoplasm (Giles et al., 2003). β-catenin acts a transcriptional switch; when it enters the nucleus, it interacts with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors to stimulate downstream target oncogenes such as *c-Myc* and *Cyclin D1* (Behrens et al., 1996; Mann et al., 1999). Wnt ligands (Wnt5a, Wnt11) also bind to the FZD family receptors, the receptor tyrosine kinase-like orphan receptor 2 (ROR2) and receptor-like tyrosine kinase (RYK) coreceptors to activate a β-catenin-independent non-canonical pathway. This signaling is mainly involved in cell polarity and migration (Lu et al., 2004a; 2004b).

Aberrant activation of the Wnt signaling pathways may be caused by β-catenin-activating mutation and *APC*-inactivating mutation. However, mutations of *CTNNB1* and *APC* are less in sporadic GC. Some recent studies suggest that epigenetic silencing of both upstream (*SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *WIF1*, *DKK1*, *DKK2*, and *DKK3*) (Ford et al., 2013; Nojima et al., 2007; Sato et al., 2007; Silva et al., 2014) and downstream (*APC* and *AXIN2*) (Li et al., 2015; Wang et al., 2012) negative regulators of Wnt signaling is also a key mechanism for pathway activation. Secreted Frizzled-related proteins (SFRPs), a family of five secreted glycoproteins, contain an extracellular cysteine-rich domain (CRD) of Frizzled (FZ) and a C-terminal domain in which they downregulate Wnt signaling by competing with FZ or binding directly to FZ (Kikuchi et al., 2012). 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-1, -2, and -4 function as antagonists of canonical Wnt signaling by binding to LRP5/6 and Kremen, thus preventing the interaction of LRP5/6 with Wnt-FZD complexes (Niehrs, 2006; Sato et al., 2007). *APC* was first identified as the gene responsible for familial adenomatous polyposis (FAP) syndrome. It is an important negative regulator of the Wnt pathway; as a component of the degradasome complex, *APC* promotes the proteasomal degradation of the Wnt effector molecule β-catenin. *APC* inactivation by hypermethylation leads to stabilization of β-catenin in the cytoplasm due to dysregulation of the β-catenin degradation (Wang et al., 2012).

Considering the complexity of the interaction of Wnt antagonists for regulating Wnt signaling, we propose that methylation of a single Wnt antagonist gene might play only a minor role in signaling activation and that the joint effect of the co-methylation of multiple antagonist genes might be more important for the activation of the signaling pathway. In the present study, we systematically and quantitatively investigated the methylation status and mRNA expression levels of six Wnt/β-catenin pathway inhibitor genes using pyrosequencing and real-time reverse-transcription polymerase chain reaction (PCR) in samples of GC tissue. We analyzed the correlation between the methylation of Wnt antagonist genes and clinical pathologic characteristics and evaluated whether quantitative methylation of Wnt antagonist genes can serve as a potential prognostic biomarker for GC. We also analyzed TCGA data to further validate the hypothesis that co-methylation of Wnt antagonist genes cooperatively drive the activation of signaling. Additionally, a demethylation drug was used to study the relationship between methylation and gene expression.

MATERIAL AND METHODS

Clinical sample collection

A total of 92 GC samples were collected from 72 male and 20 female surgical patients. These samples included 52 formalin-fixed paraffin-embedded (FFPE) samples and 40 samples of frozen GC tissue along with adjacent normal tissue. The mean age of the patients was 61.2 years (ranged 35-87). All the samples were classified by TNM (UICC 2009) staging, and 27 cases of stage I and II cancer and 65 cases of stage III and IV cancer were determined. Follow-up information about the 52 FFPE specimens was obtained from patients at the time of operation. All patients provided informed consent, and the study protocol was approved by the Ethics Committee of the Shaanxi Provincial People's Hospital. In addition, information about 262 GC samples with the methylation and mRNA expression data of six genes, as well as β-catenin expression data of matched 255 samples, were downloaded from the results of a TCGA group work (https://tcga-data.nci.nih.gov/docs/publications/stad_2014/).

DNA extraction and bisulfite modification

Genomic DNA from the samples of GC and normal adjacent tissue and FFPE samples were isolated using a Tissue DNA Kit and an FFPE DNA kit (Omega Bio-Tek, USA), respectively. Next, 1 μg DNA was bisulfite-modified using the EpiTect Fast DNA Bisulfite kit (Qiagen, Germany), according to the manufacturer's protocol.

RNA extraction and real-time PCR

Total RNA was extracted from 40 frozen samples of GC and the paired adjacent non-cancerous tissues using TRIZOL reagent (Life Technologies, USA). The quantitative mRNA expression levels were determined by real-time PCR (Applied Biosystems, Life Technologies ViiA 7 DX). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was used as an internal control. The primer sequences are shown in Supplementary Table S1. The relative amount of target RNA was calculated using the equation: relative expression level = log ($2^{-\triangle(CT(Target)-CT(reference))}$).

Pyrosequencing assays

The CpG islands of the six genes were obtained using an online software, Methprimer (http://www.urogene.org/methprimer/). A total 38 CpG dinucleotides were quantified in our study. The bisulfite-treated DNA samples were amplified by PCR in a 30-μl reaction volume, using the primers described in Supplementary Table S2. The following PCR protocol was used: 95℃ for 5 min; 40 cycles of 95℃ for 30 s,

56℃ (60℃ for *SFRP2*) for 30 s, and 72℃ for 45 s; and 72℃ for 2 min. Subsequently, sample preparation and pyrosequencing were performed as described in the Pyro-Mark MD Sample Prep Guidelines. The degree of methylation was quantified using the Pyro Q-CpG software.

Cell culture and decitabine treatment

BGC823 and MKN-45 cell lines were purchased from the Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences, Cell Resource Center. These two cell lines were incubated in an RPMI 1640 medium supplemented with 10% fetal bovine serum at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. In order to analyze gene demethylation and mRNA restoration, cells were seeded at a density of 3×10^4 cells/cm^2 in a 6-well plate and treated with 5-Aza-2'-deoxycytidine, also called decitabine (trade name Dacogen [DAC]), (2 μM and 10 μM) on days 1, 2, and 3. The drug and the medium were replaced every 24 h. Control cells were incubated without DAC.

Statistical analysis

A two-sided unpaired t test and a paired t test were performed to analyze the differences in methylation and expression levels of the genes, respectively, between GC and normal tissues. An unsupervised hierarchical clustering analysis was performed using the correlation uncentered and average linkage algorithm on Cluster 3.0, and the heatmap was constructed using TreeView. The scatter plot matrix was obtained using R software. The DAC treatment experiment was analyzed using ANOVA. Survival curves were plotted using the Kaplan-Meier method, and survival differences were determined using the log rank test. The multivariable Cox proportional hazard model was used to estimate the adjusted HR. All the statistical analyses were completed using SPSS PASW Statistics, and $p < 0.05$ was considered significant.

RESULTS

Co-methylation of Wnt antagonist genes differ significantly between GC and non-cancerous controls

In order to systematically investigate the regulatory role of epigenetic silencing of Wnt antagonists in GC, the methylation levels of *SFRP2*, *SFRP4*, *SFRP5*, *DKK1*, *DKK2*, and *APC* were determined by pyrosequencing. In 92 samples of GC tumor and 40 samples of matched normal tissue, 38 CpG dinucleotides within promoter CpG islands of these genes were analyzed. The mean methylation levels of these genes (average methylation level, lowest-highest (%)) were as follows: *SFRP2* (27.63, 9.00-53.33), *SFRP4* (20.1, 4.60-39.40), *SFRP5* (9.89, 2.00-40.16), *DKK1* (17.39, 3.67-53.83), *DKK2* (27.46, 6.29-65), and *APC* (8.83, 2.00-22.40) in the GC group; *SFRP2* (18.64, 5.56-38.44), *SFRP4* (15.13, 2.8-31.2), *SFRP5* (8.36, 1.83-20.33), *DKK1* (13.14, 5.17-24.83), *DKK2* (16.77, 3.71-36.14), and *APC* (10.44, 1.00-18.80) in the control group. Aberrant promoter methylation of *SFRP2*, *SFRP4*, *DKK1*, and *DKK2* observed in GC tissues was significantly higher than that in the control (Fig. 1). We further analyzed the co-methylation status of these genes in GC and control samples, and found that 76.1% of the GC samples contained at least two hypermethylated genes, while the co-methylation percentage in adjacent cancer

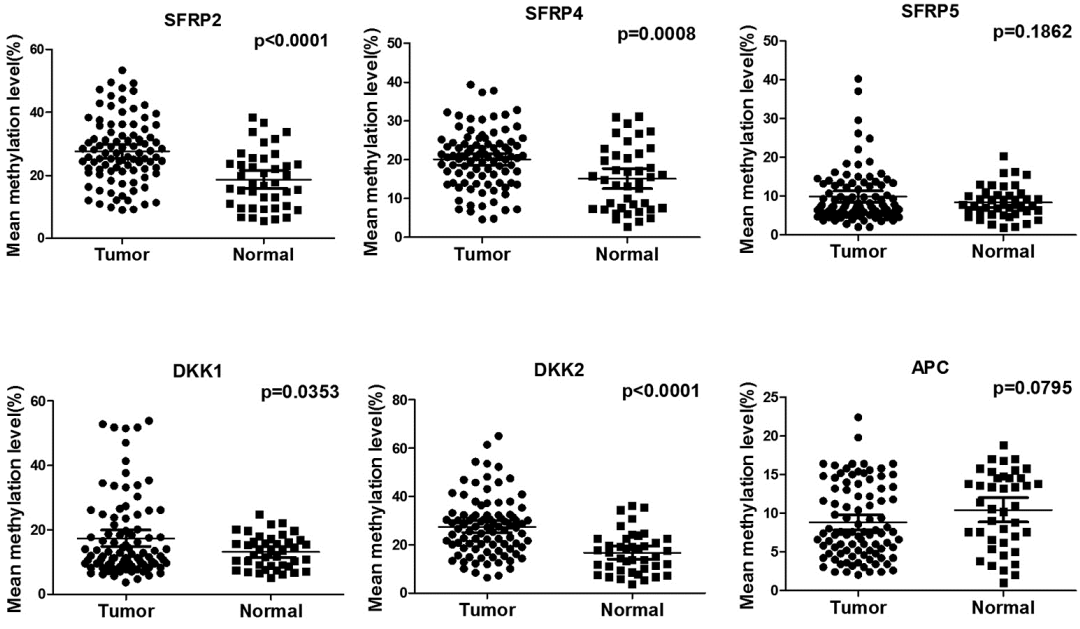

Fig. 1. Mean methylation levels of six Wnt antagonist genes in samples of gastric cancer and normal adjacent tissue. Dark spot indicates the mean methylation of each CpG site. Horizontal bar denotes the mean methylation level of each sample, and range indicates the standard error of mean.

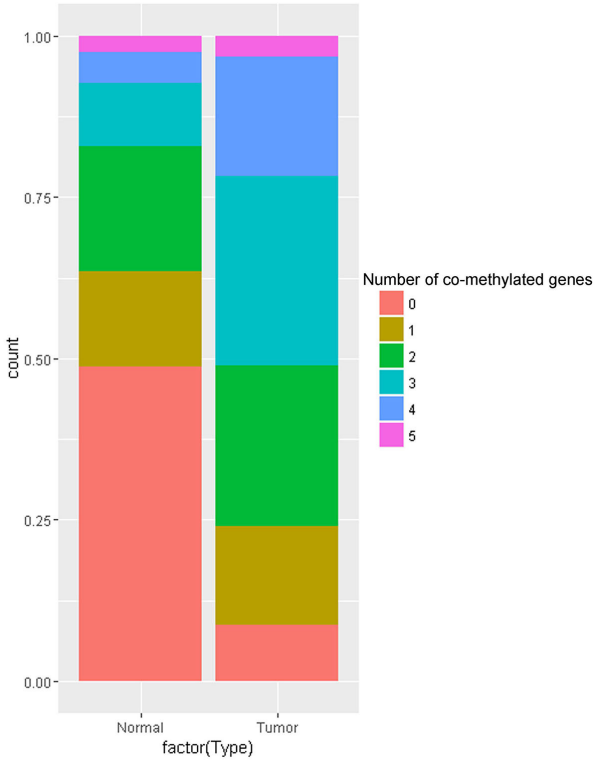

Fig. 2. Co-methylation status of Wnt antagonist genes in samples of gastric cancer and normal adjacent tissue. Each color indicates the number of co-methylated genes in samples.

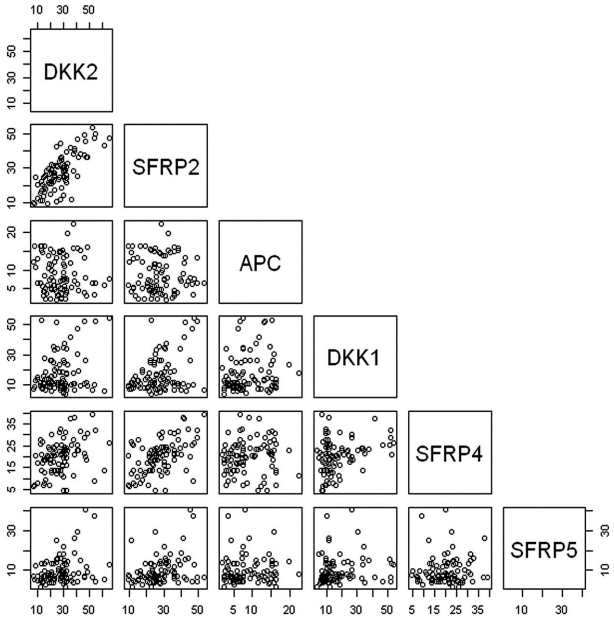

Fig. 3. Scatter plot matrix showing methylation of Wnt antagonist genes. Vacant spot represents the mean methylation level of each patient with GC. Scatter plots represent regression between each pair of genes.

tissues was only 35% (Fig. 2), suggesting that co-methylation of Wnt antagonists played an important role in tumorigenesis.

Concurrent hypermethylation of *SFRP2* and *DKK2* in GC

In order to understand the concurrent methylation status of each gene, a scatter plot matrix was prepared to compare the correlations between methylation of these Wnt antagonist genes. A significant positive correlation between *SFRP2* and *DKK2* was observed ($r = 0.751$, $p < 0.0001$) (Fig. 3, Supplementary Table S3). The unsupervised hierarchical clustering analysis revealed the overall methylation level at the CpG sites in the 92 GC samples and the 40 matched normal tissue samples. *SFRP2* and *DKK2* were clustered into one group, indicating that these two genes had similar methylation trends (Fig. 4). Taken together, these observations suggest that *SFRP2* and *DKK2* were concurrently hypermethylated.

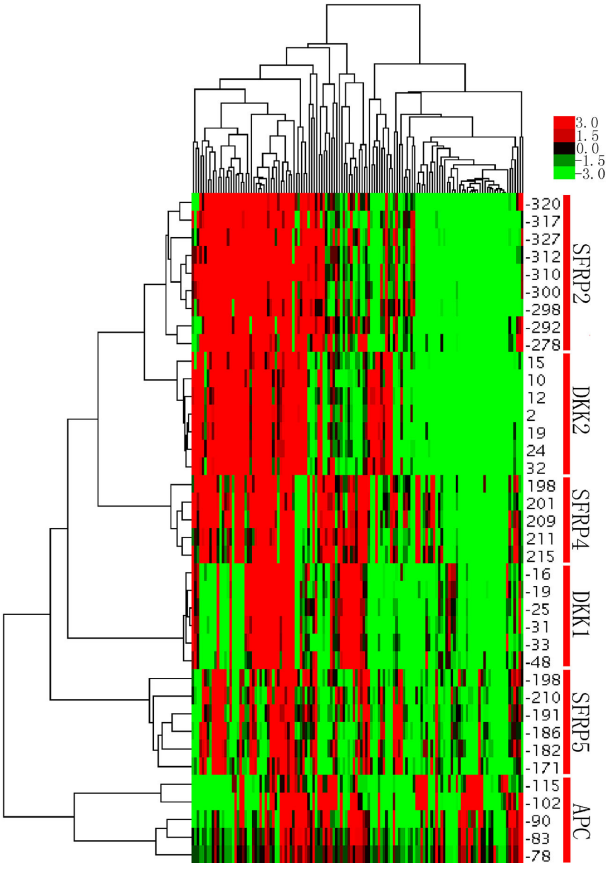

Fig. 4. Hierarchical cluster showing CpG methylation patterns of Wnt antagonist genes from samples of gastric cancer and normal tissue as assessed by pyrosequencing. Each row shows the methylation data for each of the CpG dinucleotides grouped by gene. CpG numbering represents the distance from the transcription start site. Each column represents a sample. Hierarchical clustering displays relative methylation levels (red, high methylation; green, low methylation). Concurrent hypermethylation of SFRP2 and DKK2 are highlighted on the plot.

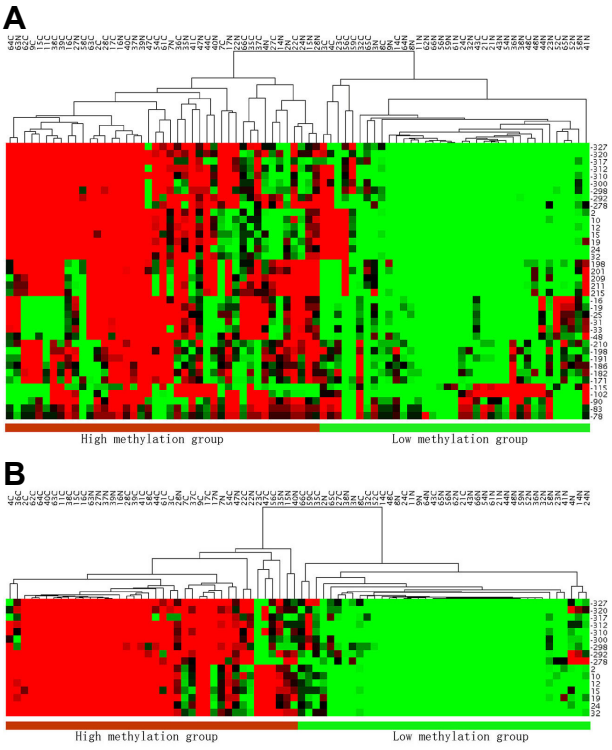

Fig. 5. Hierarchical clustering of 40 gastric cancer and their matched normal tissues. Clustering of samples by hierarchical clustering based on six genes (with 38 CpG loci) (A) and SFRP2 and DKK2 (with 16 CpG loci) (B). Better capability to distinguish between cancerous and normal samples can be seen in Fig. B.

In addition, unsupervised clustering was performed for the Wnt antagonist genes in the 40 paired samples to test whether the methylation levels of these genes could be used to distinguish between cancerous and normal samples. As shown in Fig. 5A, 37 and 43 samples were classified as low- and high-methylation group, respectively, based on these six genes (with 38 CpG loci), and 23 (62.2%) normal and 26 (60.5%) GC samples were clustered into the low- and high-methylation groups, respectively. When unsupervised clustering was performed by combining *SFRP2* and *DKK2* (with 16 CpG loci), more robust clusters were obtained (Fig. 5B). Forty samples were present in both low- and high-methylation groups. From these samples, 27(67.5%) normal and 27(67.5%) cancer samples were clustered into the low- and high-methylation group, respectively, implying that the methylation levels of *SFRP2* and *DKK2* have higher specificity to distinguish between cancer and normal samples.

Correlation between DNA methylation and gene expression levels

To determine the function of the methylation of Wnt antagonists, the mRNA expression levels of Wnt antagonist genes were assessed by quantitative PCR in 40 GC samples and matched normal controls. As expected, the mRNA expression levels of both *SFRP2* and *DKK2* were significantly lower in GC tissues than in normal tissues (Fig. 6). Regression analysis was used to explore the precise relationship between mRNA expression and methylation using data of 262 patients with GC obtained from the TCGA database. We observed a significant negative correlation between mRNA expression and methylation levels of *SFRP2* and *DKK2* (Supplementary Table S4), suggesting that hypermethylation of *SFRP2* and *DKK2* might regulate their mRNA expression level and further modulate the activation of Wnt signaling.

The association between concurrent hypermethylation of *SFRP2* and *DKK2* and β-catenin expression

The data about methylation of Wnt antagonist genes and expression levels of β-catenin were downloaded from the TCGA database to confirm our hypothesis that concurrent hypermethylation of *SFRP2* and *DKK2* drive the activation of Wnt signaling. The methylation status of the Wnt antagonists was dichotomized using the recommended cutoff (β value $>$ 0.3, as a threshold), and the relationship between methylation and β-catenin expression was analyzed. The results showed that the methylation status of single genes was not associated with β-catenin expression; however, the concurrent methylation of *SFRP2* and *DKK2* was significantly associated with increased expression of β-catenin. In addition, when the patients were classified based on the number of co-methylated genes (patients with $\leq$ 3 co-methylated genes in one group and patients with $>$ 3 co-methylated genes in another group), patients in the co-methylation group had significantly higher β-catenin levels than those in the non-co-methylation group (Fig. 7). This suggested that the concurrent silencing of multiple Wnt antagonists drive the activation of Wnt signaling.

Methylation of Wnt antagonists and clinicopathological characteristics

We next investigated the association between the methylation status of Wnt antagonist genes and the clinicopathological features of GC. The clinicopathological data of the methylated genes are summarized in Supplementary Table S5. Significant hypermethylation of *SFRP4* was observed in patients aged $>$ 60 compared with those aged $\leq$ 60 (p = 0.0037). *DKK2* methylation was significantly higher in moderately differentiated tumors than in poorly differentiated tumors (p = 0.0415). However, there were no statistically significant correlations between the methylation status of other genes and factors such as age, sex, tumor stage, lymph node metastasis, and tumor differentiation.

Overall survival analysis

To analyze the relationship between overall survival and the methylation status of Wnt antagonist genes, patients were divided into two groups according to the median methylation level of each gene. The Kaplan-Meier survival curves showed that methylation of *SFRP2* had marginally significant poor survival. Methylation of *DKK2* was significantly associated with poor overall survival (Figs. 8A and 8B), whereas the methylation status of other Wnt antagonist genes did not show any prognostic value. By using the multivariate Cox proportional hazards model, methylation status of *DKK2* (p =

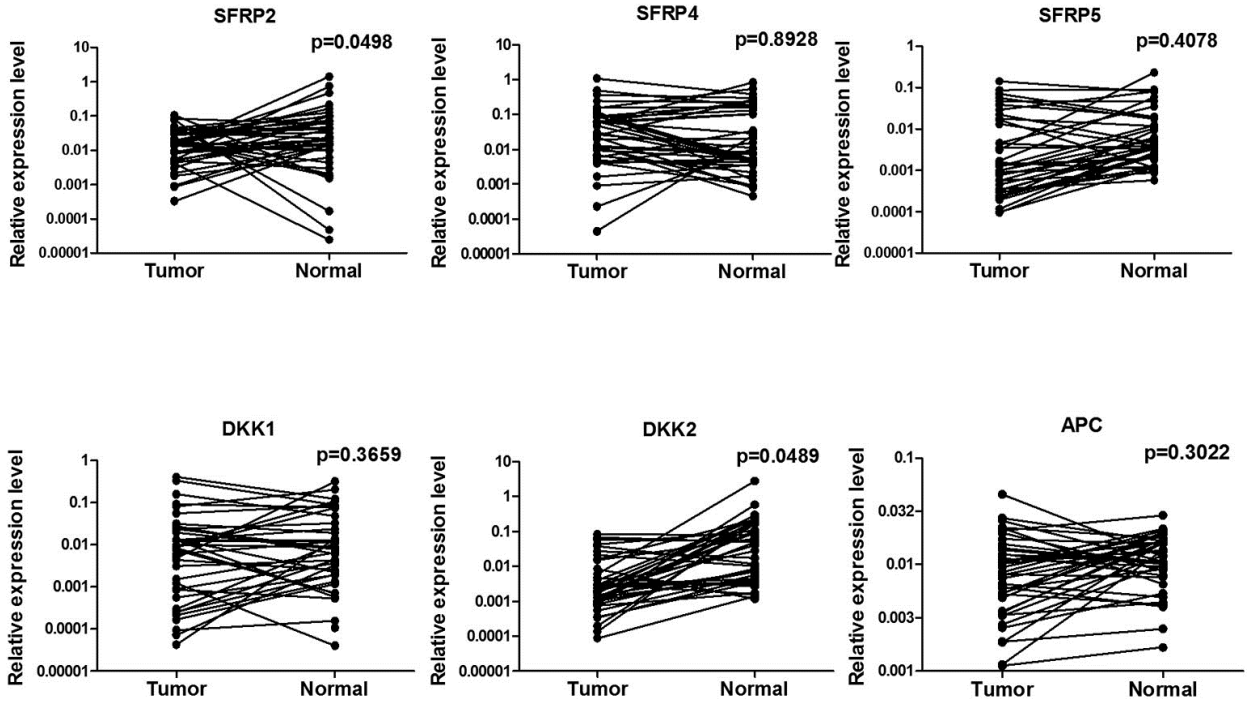

Fig. 6. mRNA expression levels of six Wnt antagonist genes in samples of gastric cancer and normal adjacent tissue. Dark spot indicates the relative mRNA expression level of each sample.

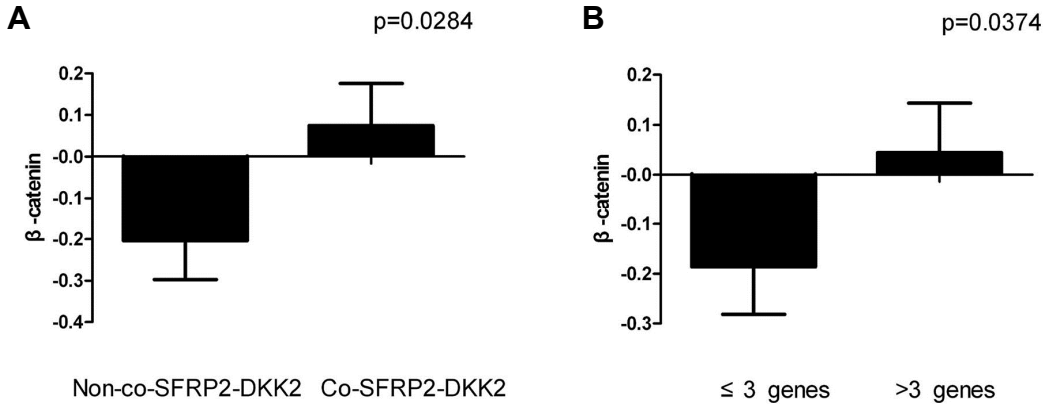

Fig. 7. Co-methylation status of Wnt antagonist genes and correlation with β-catenin. (A) Height of the histogram represents the mean level of β-catenin, and range indicates standard error of mean. Non-co-SFRP2-DKK2 group includes patients with high methylation level only in SFRP2 or DKK2 and patients with low methylation level in both SFRP2 and DKK2; Co-SFRP2-DKK2 group indicates patients with high methylation level of both two genes. (B) Patients with 0-3 highly methylated genes were placed in the ≤ 3 genes group while patients with 4-6 highly methylated genes were placed in the 〉 3 genes group.

0.041; HR = 2.051, 95% CI 1.030-4.083) was found to be an independent adverse prognostic factor (Supplementary Table S6). When concurrent methylation of *SFRP2* and *DKK2* was considered, 17 (32.7%) GC samples showed worse overall survival, as seen in Fig. 8C, supporting the idea that concurrent hypermethylation of *SFRP2* and *DKK2* might be a powerful predictor of poor prognosis.

Re-expression of SFRP2 and DKK2 mRNA after in vitro DNA demethylation

We next evaluated whether promoter methylation of Wnt antagonists was functionally associated with their mRNA expression levels in GC cell lines. To address this question, BGC823 and MKN-45 GC cell lines were treated with a methyltransferase inhibitor, Dacogen (DAC), for three days. As

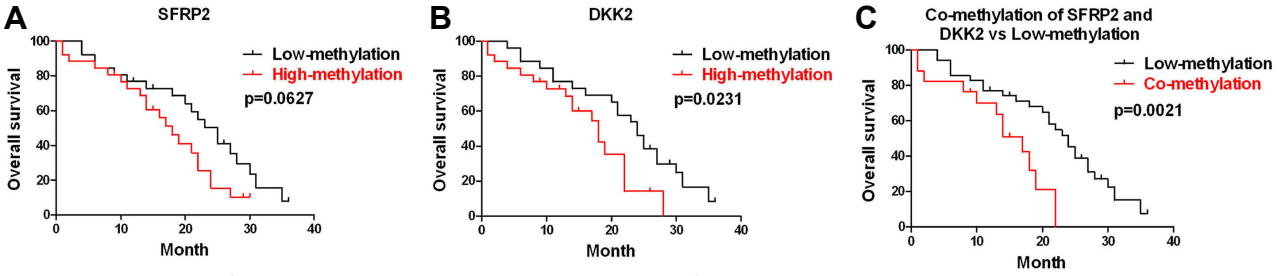

Fig. 8. Overall survival of patients correlated with promoter methylation of SFRP2 and DKK2. (A, B) Red lines indicate high methylation level of SFRP2/DKK2 promoter; dark lines indicate low methylation level of SFRP2/DKK2 promoter. (C) Survival curve plotted between co-methylation of SFRP2 and DKK2 (red line) and low methylation as well as high methylation of SFRP2 or DKK2 alone (dark line). The significance of the difference in survival was markedly enhanced by concurrent methylation of these two genes.

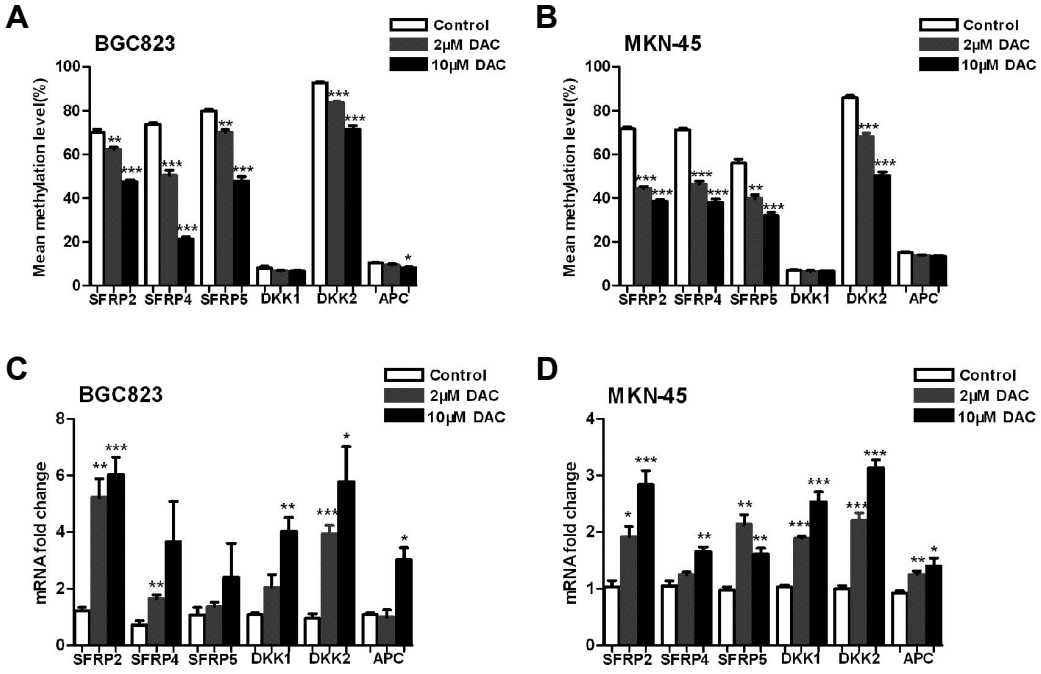

Fig. 9. Degree of methylation and expression of Wnt antagonist genes in BGC823 and MKN45 after treatment with Dacogen (DAC). (A, B) Average percentage of methylation recorded by pyrosequencing after 72 h growth in presence of 2 and 10 μM DAC. (C, D) Quantification of mRNA expression levels by PCR after 72 h growth in presence of 2 and 10 μM DAC. GAPDH was used as internal control. Three separate measurements were performed for each sample, and error bar indicates standard error of mean.

shown in Fig. 9, the DAC treatment resulted in a clear decrease in promoter methylation of *SFRP2*, *SFRP4*, *SFRP5*, and *DKK2* in a dose-dependent manner in both cell lines, and the expression of all six genes markedly increased in at least one of the two cell lines.

DISCUSSION

Constitutive Wnt/β-catenin signaling is a major contributor to gastric carcinogenesis. Ooi et al. demonstrated that Wnt/β-catenin pathways were activated in 46% of GC cases (ranged 43% to 48%) (Ooi et al., 2009). Interestingly, mutations in *APC*, *CTNNB1* (β-catenin) and *AXIN* were found to be much less common in GC (Clements et al., 2002). Woo reported that 27% (81 of 303) of GC tumors showed nuclear accumulation of β -catenin, but *CTNNB1* mutation was detected in only 5% (4 of 77) (Woo et al., 2001). Other possible mechanisms for this signaling pathway activation could be the upregulation of Wnt ligands or the modulation of gene expression by DNA methylation of promoter regions. Yoda et al. reported that genes in pathways related to cancer were more frequently affected by epigenetic alterations than by genetic alterations in GC (Yoda et al., 2015).

Although several previous studies on the methylation of Wnt antagonist genes in GC have been reported, however, how these Wnt antagonists interacted to regulate the signal-

ing pathway has not been reported. In the present study, we investigated the co-methylation of Wnt antagonist genes and its functions in Wnt signaling pathway activation in GC.

In our study, we found that *SFRP2*, *SFRP4*, *DKK1*, and *DKK2* had higher methylation levels in GC tissues than in adjacent non-cancerous tissues. The scatter plot matrix and unsupervised hierarchical clustering showed that *SFRP2* and *DKK2* were concurrently hypermethylated in GC tissues. In addition, the unsupervised hierarchical clustering showed that all the CpG sites in one gene were clustered into one group, indicating that CpG sites in regional genomes have similar methylation levels (Fig. 4). We also found that the methylation status of *SFRP2* and *DKK2* had better power to distinguish between cancerous and normal samples than that of all six genes together. This is mainly because the other genes exhibited similar levels of methylation in tumorous and normal specimens, thus affecting the clusters.

The mRNA expression data revealed that *SFRP2* and *DKK2* were expressed at lower levels in GC tissues than in adjacent non-cancerous tissues. Thus, methylation and expression of *SFRP2* and *DKK2* were correlated. Regression analysis of TCGA data revealed a significant negative correlation between mRNA expression and methylation levels in five Wnt antagonist genes (*SFRP2*, *SFRP4*, *SFRP5*, *DKK1*, and *DKK2*), strongly suggesting that methylation is responsible for the silencing of these genes. However, when we analyzed our data, we only observed a significant negative correlation between mRNA expression and DNA methylation levels for *DKK2*. Although we obtained a negative regression coefficients for the other four genes (*SFRP2*, *SFRP4*, *SFRP5*, and *DKK1*), the p values were not significant. One possible reason for this is might be that the amount of mRNA expression data used was too low to provide sufficiently powerful statistical results.

β-catenin as a key mediator of the canonical Wnt signaling pathway (Giles et al., 2003), and its accumulation in the cytoplasm/nucleus is a critical mechanism for the activation of this pathway. In this study, we analyzed the relationship between the methylation status of Wnt antagonist genes and the expression levels of β-catenin using TCGA data. When the methylation status of each gene was separately analyzed, we found no association between them. As expected, patients who showed co-methylation of *SFRP2* and *DKK2* were associated with high expression levels of β-catenin, strongly suggesting that the silencing of these two genes play a critical role in signal activation. In addition, Patients with $>$ 3 co-methylated genes showed markedly elevated β-catenin levels than those with $\leq$ 3 co-methylated genes. This result confirmed the hypothesis that the silencing of a single antagonist gene may lead to a moderate activation of Wnt signaling, but the concurrent silencing of multiple genes could significantly promote the activation of the Wnt signaling pathway.

Several studies have reported aberrant hypermethylation of Wnt inhibitor genes in GC, but have not established a correlation between methylation and clinicopathological characteristics. Hirata et al. (2009) indicated that the methylation frequency of *DKK2* was significantly associated with the grade and pathologic stage of the tumor in renal cell cancer. However, previous studies have indicated that *SFRP2* methylation was not significantly associated with any clinicopathological characteristics, including age, sex, tumor type, tumor differentiation stage, or clinical patient outcome (Cheng et al., 2007; Veeck et al., 2008). The present study shows that the methylation of *SFRP4* was significantly associated with age (p = 0.0037) and that *DKK2* methylation was significant higher in moderately differentiated than in poorly differentiated tumors (p = 0.0415). There were no significant correlations between the methylation statuses of the other genes and factors including age, sex, tumor location, tumor stage, and tumor differentiation.

Most patients with GC are diagnosed at an advanced tumor stage, where metastasis to lymph nodes has already occurred. Consequently, most patients face a poor prognosis. Therefore, it is important to identify prognostic markers that can reliably predict patient outcome. Several previous studies have demonstrated that the activation of the Wnt/β-catenin pathway and its components could indicate the clinical prognosis in GC (Ooi et al., 2009; Yu et al., 2009). In the present study, we investigated the clinical significance and prognostic value of the methylation of Wnt antagonist genes in 52 patients. We found that a high methylation rate of *DKK2* was significantly associated with poor overall survival; the multivariate Cox proportional hazards model revealed that methylation of *DKK2* (p = 0.041; HR = 2.051, 95% CI 1.030-4.083) was an independent adverse prognostic factor. *SFRP2* was weaker associated with poor overall survival. Interestingly, for samples in which both *SFRP2* and *DKK2* were methylated, the significance of the survival difference was markedly enhanced. Thus, concurrent methylation of these two genes might be a useful prognostic marker. This observation also strengthened the hypothesis that co-methylation of *SFRP2* and *DKK2* activates the Wnt signaling pathway, resulting in deterioration of the prognosis of patients.

We confirmed that the expression levels of all six Wnt antagonist genes were restored after DAC treatment in at least one of the two cell lines. Four of six genes (*SFRP2*, *SFRP4*, *SFRP5*, and *DKK2*) exhibited high methylation levels and two genes (*DKK1* and *APC*) showed a low methylation level in both cell lines, the methylation levels in most genes were consistent with those in our clinical samples. Although *DKK1* and *APC* were not sensitive to DAC treatment, the change in mRNA expression levels induced by incubation with DAC was remarkable. Previous studies have reported that *APC* had two promoters, 1A and 1B. Quantitative methylation analyses showed that promoter 1A was methylated in cancerous as well as non-cancerous gastric mucosae, while promoter 1B was not methylated in any of the samples (Hosoya et al., 2009; Tsuchiya et al., 2000). Our results indicate that another promoter region might regulate the mRNA expression.

In summary, our results show that the methylation levels of several Wnt antagonist genes significantly increased in GC, while their mRNA expression levels decreased. Concurrent hypermethylation of *SFRP2* and *DKK2* cooperatively promoted activation of the Wnt signaling pathway. We demonstrated that high levels of methylation of *DKK2* are inde-

pendent predictors of a poor prognosis in patients with GC. Consideration of *SFRP2* and *DKK2* methylation status together might be a powerful prognostic marker for GC.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by the Program for New Century Excellent Talents in University to Penggao Dai (NECT-12-1048) and the National Natural Science Foundation of China (31271353).

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