Original Article

Promoter Methylation of the Wnt/β-Catenin Signaling Antagonist *Dkk-3* Is Associated With Poor Survival in Gastric Cancer

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BACKGROUND: Abnormal activation of the Wnt/β-catenin signaling pathway is common and critical in the pathogenesis of digestive cancers. In this study, the authors investigated the promoter methylation of the dickkopf homolog 3 gene *Dkk-3* in these cancers and its prognostic significance in gastric cancer. **METHODS:** *Dkk-3* methylation was assessed in 173 patients with gastric cancers (including 104 patients who were followed for up to 4090 days) and in 128 patients with colorectal cancer. Cell growth was evaluated by using a colony-formation assay. For survival analyses, the authors used Kaplan-Meier plots, the log-rank test, and Cox proportional regression. **RESULTS:** *Dkk-3* was silenced or down-regulated in 12 of 17 gastric cancer cell lines (70.6%) and in 3 of 9 colon cancer cell lines (33.3%). The loss of gene expression was associated with promoter methylation, which could be restored by demethylating agents. Ectopic expression of *Dkk-3* suppressed colony formation. Moreover, methylation of *Dkk-3* was detected in 117 of 173 primary gastric tumors (67.6%) and in 67 of 128 colorectal tumors (52.3%). The clinical significance and the prognostic value of *Dkk-3* methylation also were examined in 104 gastric cancers and in 84 colorectal cancers. Multivariate analysis indicated that *Dkk-3* methylation was associated significantly and independently with poor disease survival (relative risk, 2.534; 95% confidence interval, 1.54–4.17; $P = .002$) in gastric cancer, but not in colorectal cancer. Kaplan-Meier survival curves revealed that patients who had *Dkk-3* methylated gastric cancers had a significantly shorter survival (median, 0.76 years) compared with patients who did not have *Dkk-3* methylation (median, 2.68 years; $P < .0001$; log-rank test). **CONCLUSIONS:** Epigenetic silencing of the *Dkk-3* gene by promoter methylation was a common event in gastric cancer and was associated with a poor outcome in such patients. **Cancer 2009;115:49–60. © 2008 American Cancer Society.**

KEY WORDS: dickkopf homolog 3, tumor-suppressor gene, digestive tumors, methylation, prognosis.

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Digestive cancers account for about 20% of all cancers and are among the leading causes of cancer mortality in the world. Their incidence varies greatly in different parts of the world and among various ethnic groups.[1,2] It is recognized increasingly that epigenetic inactivation of tumor-related genes by promoter methylation plays a crucial role in the development of digestive malignancies. Methylation in cancer has significant tissue specificity. For example, the mutL homolog 1 gene *MLH1* is methylated in colorectal cancers[3] and gastric cancers[4] but is methylated infrequently in hepatocellular carcinomas (HCC).[5] It is believed that this difference in methylation is related to a tissue-specific, selective advantage imparted by the methylation events; however, it also may be related to tissue-specific exposures.[5]

Abnormal activation of the Wnt/β-catenin signaling pathway is common and is critical in the pathogenesis of digestive cancers. The Wnt pathway consists of highly conserved, secreted ligands that bind cell-surface receptors called frizzled and lipoprotein receptor-related proteins.[6,7] The Wnt signaling leads to stabilization and nuclear translocation of β-catenin, which, in turn, forms a complex with T-cell factor (TCF) transcription factors to activate target genes. Many of these target genes are involved in pathogenesis and tumorigenesis.[8,9] Genetic alterations of adenomatous polyposis coli (APC) and β-catenin, regulators in the Wnt signaling pathway have been observed in human colon cancer,[10] gastric cancer,[11,12] and HCC.[13] These findings provide evidence that the activation of the Wnt signaling pathway is related to the development of digestive cancers. Conversely, several antagonists of Wnt signaling have been identified with 2 functional classes: the secreted frizzled-related protein (sFRP) class and the dickkopf (Dkk) class.[14] Once Wnt signaling is suppressed by Dkk, β-catenin is phosphorylated and subsequently targeted for ubiquitination and degradation. Conversely, the functional loss of *Dkk* can contribute to the activation of the Wnt signaling pathway and has a tumor-promoting effect through the dysregulation of cell proliferation and differentiation. Therefore, it is hypothesized that that members of the *Dkk* class of genes are tumor-suppressor genes: *Dkk-1* reportedly functions as a suppressor of colon cancer cell transformation.[15] The *Dkk-3* gene, located on chromosome 11p15.1, is repressed in a variety of human cancer cell lines and in various types of human cancers,[16–21] which also makes it a candidate tumor-suppressor gene. Several lines of evidence suggest that *Dkk-3* down-regulation in cancers is caused by promoter methylation.[20,22–24] In line with these observations, we also were able to identify *Dkk-3* silencing in colon cancer cell lines using methylated CpG island amplification combined with representational difference analysis. However, reports about the prognostic value of epigenetic silencing of *Dkk-3* in cancer samples and its clinical implications are rather scarce. We initiated this study to explore the possible role of *Dkk-3* in gastric and colorectal cancer and its potential relevance to clinical outcome. We were able to demonstrate that loss of *Dkk-3* expression by promoter methylation is common in gastric and colorectal cancers. We also demonstrated that *Dkk-3* promoter methylation in primary gastric cancer correlates strongly with a poor patient prognosis and, in multivariate analyses, is an independent prognostic marker of survival in patients with gastric cancer.

MATERIALS AND METHODS

Tumor Cell Lines, Primary Tumor Samples, and Normal Tissue Samples

A series of human tumor cell lines from the digestive system were used, including 17 gastric cancer cell lines (Kato III, YCC1, YCC2, YCC3, YCC6, YCC7, YCC9, YCC10, YCC11, YCC16, SNU719, MKN28, MKN45, SNU1, SNU16, NCI87, and AGS) and 8 colon cancer cell lines (HCT116, DKO, HT-29, Lovo, SW480, SW48, DLD1, and CaCO2). Human tumors, including 173 gastric cancers (104 with follow-up information) and 128 colorectal cancers (84 with follow-up information), were obtained from patients at the time of operation. In addition, 20 age- and sex-matched samples of normal gastric mucosa were included as controls. The samples immediately were snap frozen in liquid nitrogen and stored at −80°C. The remaining tissue specimens were fixed in 10% formalin and embedded in paraffin for routine histologic examination. All patients provided informed consent for obtaining the study specimens. The study protocol was approved by the Ethics Committee of the Chinese University of Hong Kong. Human normal adult and fetal tissue RNA samples were purchased commercially.[25]

RNA Extraction and Semiquantitative Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was extracted from cell pellets using TriReagent (Molecular Research Center, Inc., Cincinnati, Ohio). Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described previously using the Go-Taq polymerase system (Promega, Madison, Wis) and the GeneAmp RNA PCR system (Applied Biosystems, Foster City, Calif) with glyceraldehyde 3-phosphate dehydrogenase as a control. The following *Dkk-3*–specific primers were used: forward, 5′-CACCCTCAAT GAGATGTTCC-3′; and reverse, 5′-TGGTCTCATTG TGATAGCTG-3′. The RT-PCR protocol was 32 cycles with an annealing temperature of 55°C.

DNA Extraction and Methylation-Specific Polymerase Chain Reaction Analysis

Genomic DNA was extracted from the cell pellets and tissues using TriReagent or the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). DNA was modified chemically with sodium metabisulphite. The bisulfite-modified DNA was amplified by using primer pairs that specifically amplify either methylated or unmethylated sequences of the *Dkk-3* genes. The following methylated *Dkk-3*–specific primers were used: sense, 5′-TTTCGGGTATC GGCGTTGTC-3′ (positions +176 to +195 from the transcriptional start site); and antisense, 5′-ACTAAACC GAATTACGCTACG-3′ (positions +323 to +303). The following unmethylated *Dkk-3*–specific primers were used: sense, 5′-GTTTTTTTGGGTATTGGTGT TGTT-3′ (positions +172 to +195); and antisense, 5′-CAACTAAACCAAATTACACTACA-3′ (positions +325 to +303). These primer pairs have been tested previously and do not amplify any bisulphite-untreated DNA. Methylation-specific PCR (MSP) was performed for 40 cycles using Taq-Gold polymerase (Applied Biosystems).

5-Aza-2′-Deoxycytidine and Trichostatin A Treatment

Cells were seeded at a density of 1×10^6 cells/mL. After overnight culture, cells were treated with 10 mM of the DNA demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC) (Sigma Chemical Company, St. Louis Mo) for 72 hours, with 300 nmol/L of trichostatin A (Sigma) for additional 24 hours, and then were harvested.

Construction of Dkk-3 Expression Vector and in Vitro Translation

The *Dkk-3* expression vector was generated by PCR cloning with the pcDNA3.1 TOPO TA Expression Kit (Invitrogen). Combinational DNA corresponding to full-length *Dkk-3* was obtained by RT-PCR amplification of normal human thymus RNA (Clontech, Palo Alto, Calif) with primers unique for *Dkk-3*. PCR aliquots were subcloned in the pcDNA3.1 TOPO vector. Clones were screened and sequenced using vector-specific primers.

Colony Formation Assay

Cells from the gastric cancer cell line MKN45 (2×10^5 cells per well) were transfected with 0.5 μg *Dkk-3*–expressing or empty vector (pcDNA3.1) using FuGENE 6 (Roche). Transfected cells were selected with G418 sulfate (0.4 mg/mL) (Merck, Darmstadt, Germany) for 2 weeks. Colonies were fixed with methanol/acetone (1:1), stained with Gentian Violet, and counted. All experiments were performed in triplicate wells 3 times each.

Statistical Analysis

The chi-square test was used to compare the frequency of methylation in tumor and patient characteristics and distributions of methylation by vital status. Patients' ages (at the start of follow-up) by vital status were compared by using a *t* test. The relative risk (RR) of death associated with *Dkk-3* methylation and other predictor variables were estimated first from a univariate Cox proportional hazards model. Multivariate Cox models also were constructed to estimate the RR for *Dkk-3* methylation with adjustments for potential confounding risk factors. Overall survival in relation to methylation status was evaluated by using Kaplan-Meier survival curves and log-rank tests. All analyses were performed using SAS for Windows, version 9 (SAS Institute, Inc., Cary, NC). $P < .05$ was considered statistically significant.

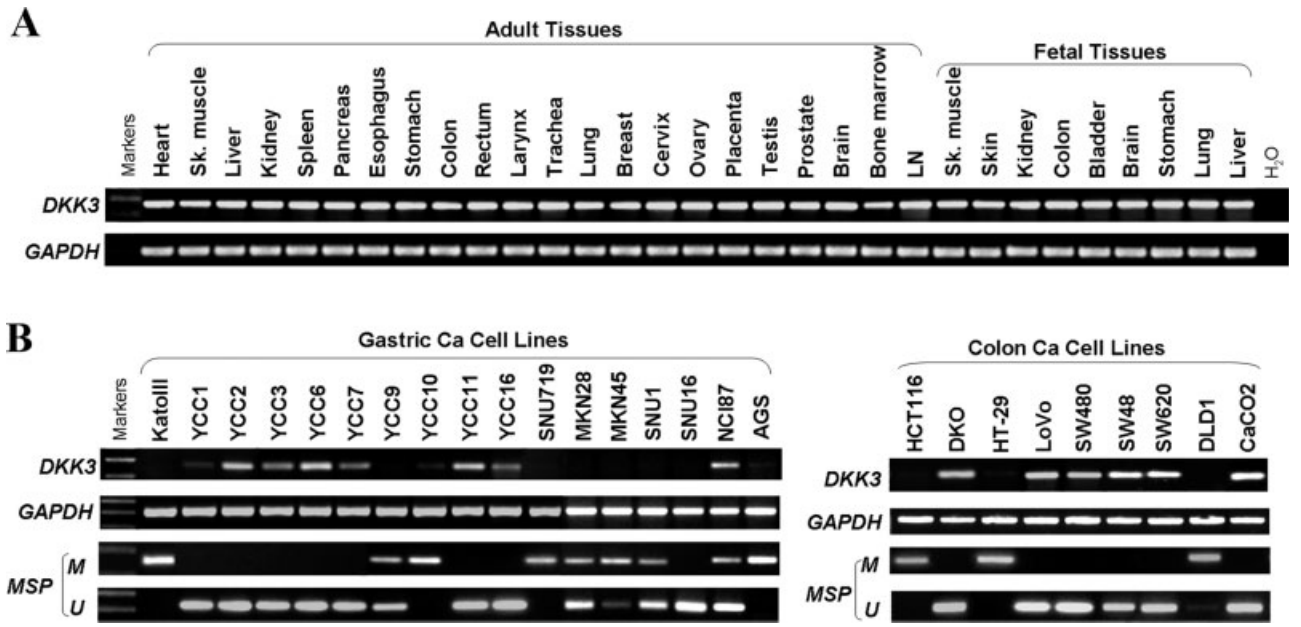

FIGURE 1. (A) Messenger RNA (mRNA) expression of the dickkopf homolog 3 gene *Dkk-3* in human normal adult tissue and fetal tissues by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) used as the control. (B) Representative *Dkk-3* mRNA expression by semiquantitative RT-PCR and promoter methylation of the *Dkk-3* gene by methylation-specific PCR (MSP) in multiple tumor cell lines. Sk. indicates skeletal; LN, lymph node; U, unmethylated; M, methylated.

RESULTS

Correlation of Dkk-3 *Expression Levels With Methylation Status in Digestive Tumor Cell Lines*

We compared the expression profiles of HCT116 and HCT116-DKO cells by using Affymetrix microarray expression analysis. *Dkk-3* is 1 in a group of up-regulated genes detected in HCT116-DKO cells compared with HCT116 cells. Because *Dkk-3* is expressed widely in normal adult and fetal tissues (Fig. 1A), we also screened its expression levels in a series of tumor cell lines from the digestive system by using semiquantitative RT-PCR. *Dkk-3* expression was reduced or silenced in 13 of 18 gastric cancer cell lines (72.2%) and in 3 of 9 colon cancer cell lines (33.3%) (Fig. 1B). Next, we examined the role of methylation in the silencing of *Dkk-3*. By using MSP, we detected *Dkk-3* methylation in all cell lines that had silenced or low expression, whereas unmethylated promoter was detected in the cell lines that expressed *Dkk-3* (Fig. 1B).

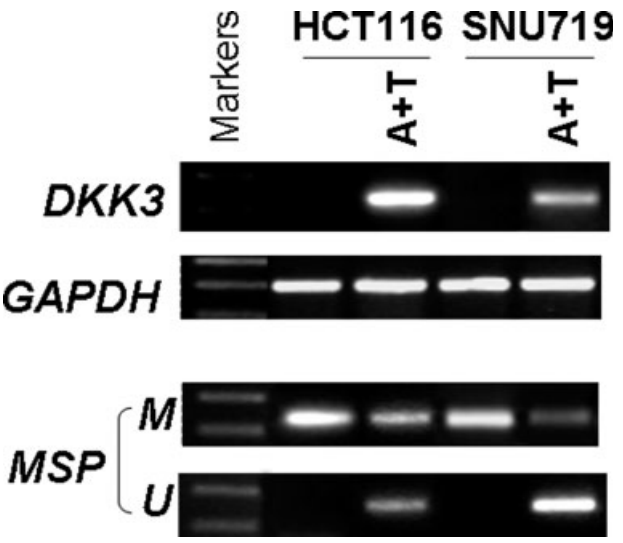

FIGURE 2. Expression of the dickkopf homolog 3 gene *Dkk-3* could be restored in tumor cells by treatment with 5-Aza-2′-deoxycytidine (A) and trichostatin A (T). Two highly methylated and silenced cell lines from gastric cancer (SNU719) and colon cancer (HCT116) were treated. Up-regulated expression and demethylation (shown by the unmethylated [U] bands) were detected. MSP indicates methylation-specific polymerase chain reaction; M, methylated.

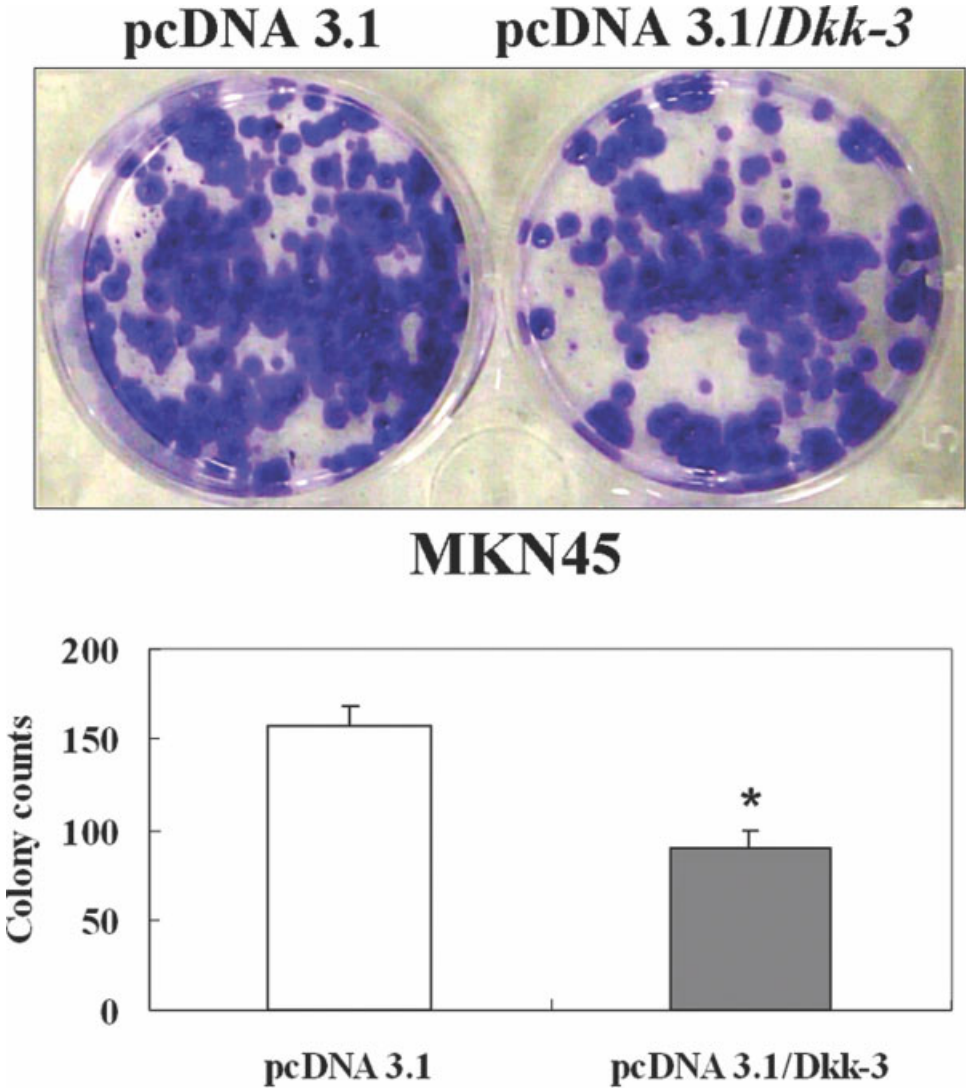

FIGURE 3. The dickkopf homolog 3 gene *Dkk-3* inhibited tumor cell colony formation. MKN45 cells were transfected with *Dkk-3*–expressing cells or with empty vector (pcDNA3.1) and were maintained in the presence of G418 sulfate for 2 weeks. Assays were performed in triplicate. Quantitative analyses of colony numbers are shown as the mean values ± standard deviation. *P* values were calculated using the Student *t* test. The asterisk indicates a statistically significant difference ($P = .002$).

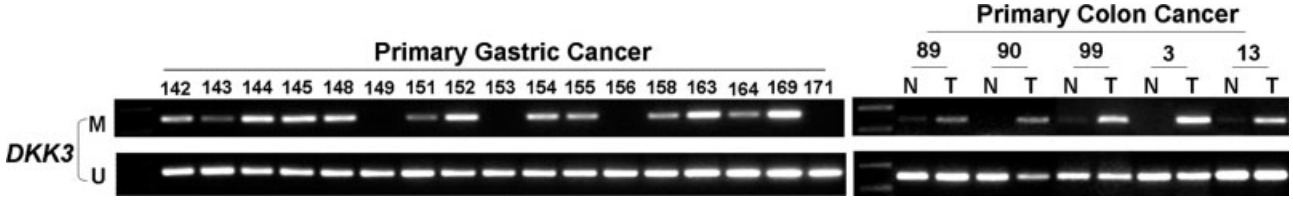

FIGURE 4. Representative methylation-specific polymerase chain reaction analysis results from primary tumors. Dkk3 indicates dickkopf homolog 3; M, methylated; U, unmethylated; N, normal tissues; T, tumor tissues.

Dkk-3 *Expression Could Be Restored With 5-Aza-Deoxycytidine and Trichostatin A Treatment in Cancer Cell Lines*

To assess whether methylation of *Dkk-3* promoter was associated with transcriptional silencing, we analyzed *Dkk-3* messenger RNA (mRNA) expression in the presence or absence of the demethylating agent 5-Aza-dC combined with trichostatin A in 3 heavily methylated and silenced cell lines from colon cancer (HCT116) and gastric cancer (SNU719). *Dkk-3* expression was restored after demethylation treatment in both cell lines (Fig. 2), suggesting that CpG methylation contributed directly to the silencing of *Dkk-3* in tumor cells.

Table 1. Methylation Status of the Dickkopf Homolog 3 Gene *Dkk-3* in Tumors and Adjacent Nontumor Tissues

	No./Total No. (%)		
Origin	Tumor	Nontumor	*P*
Gastric cancer	117/173 (67.6)	36/104 (34.6)	<.0001
Colorectal cancer	67/128 (52.3)	1/20 (5)	.005

Dkk-3 *Suppresses Tumor Cell Growth*

The frequent silencing of *Dkk-3* by methylation in multiple gastric and colon cancer cell lines suggests that *Dkk-3* is likely a tumor suppressor. To this end, we expressed an exogenous *Dkk-3* gene in MKN45 cells in which the endogenous gene is silenced by promoter methylation. We performed colony formation assays using MKN45 cells that were transfected with a *Dkk-3* gene construct (pcDNA3.1/*Dkk-3*) or with an empty vector (pcDNA3.1). The numbers of colonies formed were counted after 2 weeks in culture with G418 selection. We observed that *Dkk-3* re-expression decreased markedly the numbers of colonies (Fig. 3).

Dkk-3 *Frequently Is Hypermethylated in Primary Gastric and Colorectal Cancers*

Having established that aberrant promoter methylation in the *Dkk-3* gene was correlated with transcriptional silencing, we investigated *Dkk-3* methylation in primary gastric and colorectal cancers. Aberrant methylation was detected in 67.6% (117 of 173) of gastric cancers and in 52.3% (67

Table 2. Clinicopathologic Features of Patients With Gastric Cancer According to Methylation Status of the Dickkopf Homolog 3 Gene *Dkk-3* Promoter

	Methylated (n = 64)		Nonmethylated (n = 40)		
Variable	No.	%	No.	%	*P*
Age: Mean ± SD, y	66.7 ± 12.7		64 ± 13.3		.293
Sex					.400
Men	33	57.9	24	42.1	
Women	31	66	16	34.04	
***H. pylori* infection**					.79
Positive	17	51.5	16	48.5	
Negative	17	54.8	14	45.2	
Lauren type					.109
Diffuse	22	71	9	29	
Intestinal	22	52.4	20	47.6	
Differentiation					.242
Poor (or no differentiation)	23	54.8	19	45.2	
Well or moderate	41	66.1	21	33.9	
TNM classification					.007
I	7	46.7	8	53.3	
II	6	33.3	12	66.7	
III	22	71	9	29	
IV	22	78.6	6	21.4	

SD indicates standard deviation.

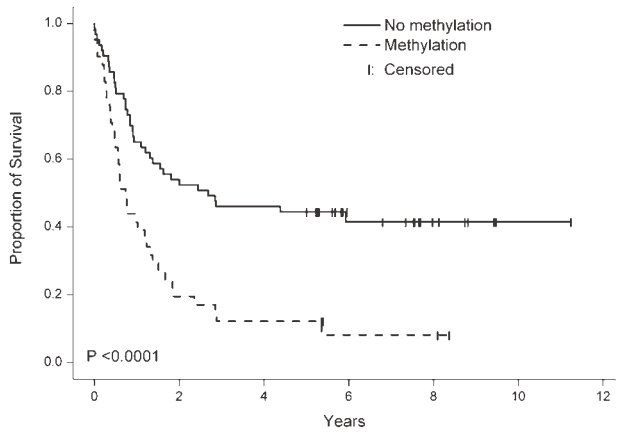

FIGURE 5. Methylation status of the dickkopf homolog 3 gene *Dkk-3* predicted clinical outcome in patients with gastric cancer. Kaplan-Meier analysis of survival was performed according to the presence of *Dkk-3* promoter methylation in 104 patients with gastric cancer. Patients who had tumors with *Dkk-3* methylation had poorer survival compared with patients who had tumors without *Dkk-3* methylation.

of 128) of colorectal cancers. Illustrative examples are shown in Figure 4. Promoter methylation of *Dkk-3* also was detected in some paired adjacent nontumor tissues, and methylation was significantly more common in tumor tissues than in adjacent nontumor tissues (Table 1).

Dkk-3 *Is a Predictor of Poor Outcome in Gastric Cancer*

Because a high prevalence of *Dkk-3* methylation was demonstrated in gastric cancer, the association of *Dkk-3* methylation status with clinicopathologic characteristics, including clinical outcome, was analyzed in 104 gastric cancer patients with known survival data (median survival, 503 days; range, 4–4090 days). Methylation of *Dkk-3* was observed more frequently in patients with advanced tumor stages than in patients with less advanced tumor stages ($P = .007$). However, there were no correlations between *Dkk-3* methylation status and age, sex,

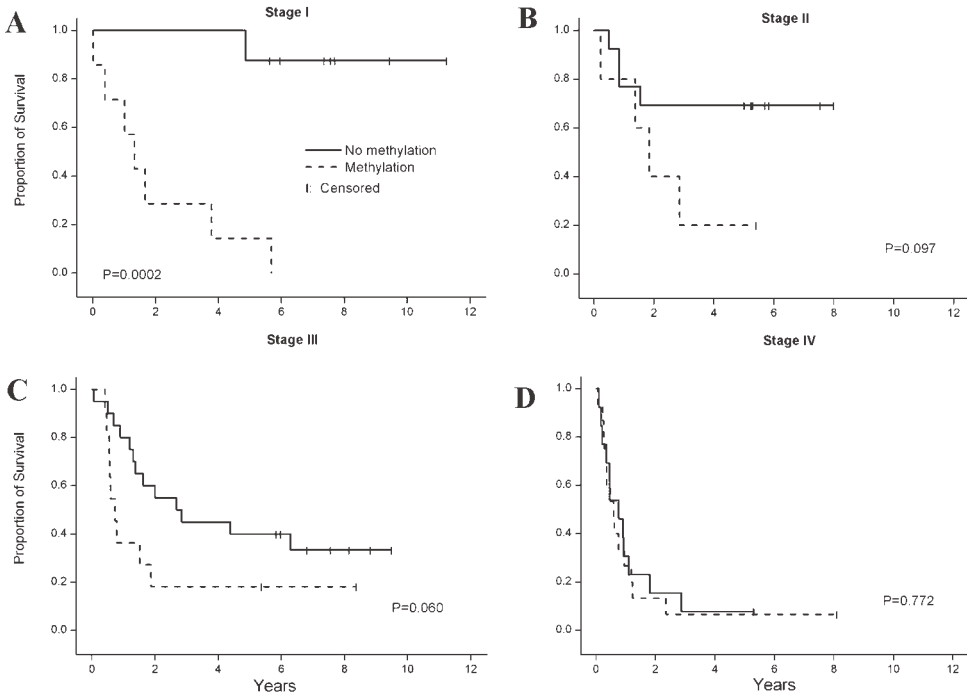

FIGURE 6. Kaplan-Meier survival curves according to methylation status of the dickkopf homolog 3 gene *Dkk-3* in patients with gastric cancer. Patients with and without *Dkk-3* methylation were compared. (A) Patients who had TNM stage I disease exhibited significantly poorer survival. (B, C) Patients who had TNM stage II and III patients had marginally significant poorer survival. (D) Patients who had TNM stage IV disease did not differ significantly.

Table 3. Distribution of Selected Patient Characteristics by Survival Status

	Alive (n = 31)		Dead (n = 73)		
Variable	**No.**	**%**	**No.**	**%**	***P***
Age: Mean ± SD, y	63.4 ± 12.7		66.6 ± 13		.262
Sex					.195
Men	20	35.1	37	64.9	
Women	11	23.4	36	76.6	
***H. pylori* infection**					.846
Positive	12	36.4	21	63.6	
Negative	12	38.7	19	61.3	
Lauren type					.055
Intestinal	17	40.5	25	59.5	
Diffuse	6	19.3	25	80.7	
Differentiation					.518
Poor (or no differentiation)	14	33.3	28	66.7	
Well or moderate	17	27.4	45	72.6	
TNM classification					.002
I	7	46.7	8	53.3	
II	10	55.6	8	44.4	
III	9	29.0	22	71.0	
IV	2	7.1	26	92.9	
***Dkk-3* methylation**					<.0001
Yes	5	7.8	59	92.2	
No	26	65.0	14	35	

SD indicates standard deviation; *Dkk-3*, indicates the dickkopf homolog 3 gene.

Helicobacter pylori infection, Lauren type, or tumor differentiation (Table 2).

The Kaplan-Meier estimator of the survivorship function was used to determine the effects of *Dkk-3* methylation on patient survival. The overall survival of gastric cancer patients with and without *Dkk-3* methylation was compared by using the log-rank test. The overall prognosis for patients who had tumors with methylated *Dkk-3* was significantly worse than the prognosis for patients who had tumors without methylation ($P < .0001$; log-rank test) (Fig. 5). The data were stratified further according to the TNM tumor classification, because this is an independent risk factor in patients with gastric cancer. The Kaplan-Meier survival curves demonstrated that the survival of patients who had TNM stage I tumors with *Dkk-3* methylation was significantly shorter compared with the survival of patients who had stage I tumors without methylation (Fig. 6). A similar trend also was noted in patients with stage II and III tumors (Fig. 6).

Table 3 summarizes the distribution of the clinicopathologic characteristics between patients with gastric cancer who remained alive and those who died. The disease survival status indicates that advanced TNM tumor stage and *Dkk-3* methylation were associated significantly with vital status ($P = .002$ and $P < .0001$, respectively), However, the histologic tumor type according to Lauren was associated marginally with vital status ($P = .055$), whereas age, sex, *H. pylori* infection, and tumor differentiation did not have any prognostic value within the available follow-up period. In the univariate Cox regression analysis, *Dkk-3* methylation was associated with a highly significantly increased risk of cancer-related death (RR, 6.40; 95% CI, 3.40-12.1; $P < .0001$). Lauren type and tumor stage also were significant predictors of patient outcome (Table 4). Multivariate analysis indicated that *Dkk-3* methylation was associated with higher mortality (RR, 1.73; 95% CI, 1.03–2.92; $P = .02$) (Table 5). Multivariate analysis revealed that the tumor stage also was associated with prognosis (Table 5).

Table 4. Univariate Cox Regression Analysis of Potential Prognostic Factors for Patients With Gastric Cancer

Variable	RR (95% CI)	*P*
Age	1.01 (0.99–1.03)	.307
Sex		
Men	0.65 (0.41–1.03)	.064
Women	1.00	
***H. pylori* infection**		
Positive	1.14 (0.61–2.11)	.688
Negative	1.00	
Lauren type		
Diffuse	1.78 (1.02–3.11)	.044
Intestinal	1.00	
Differentiation		
Poor or no differentiation	0.92 (0.57–1.47)	.719
Well or moderate	1.00	
TNM classification		
I	0.24 (0.11–0.55)	.0006
II	0.21 (0.09–0.46)	.0001
III	0.41 (0.23–0.72)	.0022
IV	1.00	
***Dkk-3* methylation**		
Yes	6.40 (3.40–12.1)	<.0001
No	1.00	

RR indicates relative risk; 95% CI, 95% confidence interval; *Dkk-3*, the dickkopf homolog 3 gene.

Table 5. Multivariate Cox Regression Analysis of Potential Prognostic Factors for Patients With Gastric Cancer

Variable	RR (95% CI)	*P*
Age	1.00 (0.98–1.03)	.971
Sex		
Men	0.53 (0.26–1.06)	.073
Women	1.00	
Lauren type		
Diffuse	1.16 (0.61–2.20)	.658
Intestinal	1.00	
TNM classification		
I	0.16 (0.05–0.51)	.002
II	0.22 (0.09–0.53)	.001
III	0.49 (0.23–1.04)	.062
IV	1.00	
***Dkk-3* methylation**		
Yes	1.73 (1.03–2.92)	.020
No	1.00	

RR indicates relative risk; 95% CI, 95% confidence interval; *Dkk-3*, the dickkopf homolog 3 gene.

Table 6. Multivariate Cox Regression Analysis of Potential Prognostic Factors for Patients With Colorectal Cancer

Variable	RR (95% CI)	*P*
Age	1.02 (0.99–1.05)	.211
Sex		
Men	1.37 (0.69–2.74)	.367
Women	1.00	
TNM classification		
I and II	0.21 (0.08–0.54)	.001
III	0.62 (0.31–1.27)	.193
IV	1.00	
***Dkk-3* methylation**		
Yes	1.44 (0.75–2.77)	.272
No	1.00	

RR indicates relative risk; 95% CI, 95% confidence interval; *Dkk-3*, the dickkopf homolog 3 gene.

The relation between *Dkk-3* methylation and prognosis in 84 patients with colorectal cancer also was analyzed. Multivariate Cox regression analysis (Table 6) was conducted to measure the hazard ratio after controlling for potential confounding factors such as age, sex, TNM classification, and *Dkk-3* methylation. Patients who had stage I and II disease had a significantly longer survival compared with patients who had stage IV disease ($P = .001$). However, there were no significant overall associations between the methylation status of *Dkk-3* and survival (Table 6). Also in the Kaplan-Meier analysis, the overall survival of patients who had colorectal cancer with *Dkk-3* methylation did not differ significantly from that of patients without *Dkk-3* methylation (Fig. 7).

DISCUSSION

In the current study, we demonstrated that *Dkk-3* transcription frequently is reduced or silenced in gastric and colon cancer cell lines. This finding supports the recent observations made by other groups using digestive cancer cell lines.[16,26] *Dkk-3* is located on chromosome 11p15, and Pfeifer observed that this region is an important target of methylation-mediated genetic imprinting.[27] Our study revealed that silenced or diminished expression of the *Dkk-3* gene is associated closely with promoter methylation. The inhibition of DNA-methyltransferase activity by 5-Aza-dC leads to a reversion of methylation and the re-expression of the silenced gene. Because transcriptional

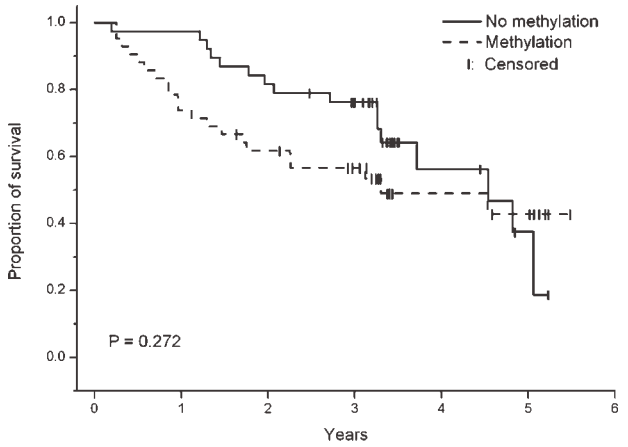

FIGURE 7. Kaplan-Meier survival curve according to methylation status of the dickkopf homolog 3 gene *Dkk-3* in patients with colorectal cancer. Among 84 colorectal cancer patients, patients who had tumors with *Dkk-3* methylation did not differ significantly compared with patients who did not have tumors with *Dkk-3* methylation.

silencing mediated by methylation involves the recruitment of histone deacetylase activity,[28] the effect of 5-Aza-dC is augmented by the histone deacetylase inhibitor trichostatin A. *Dkk-3* gene mRNA expression was increased in silenced cell lines after treatment with 5-Aza-dC and trichostatin A, indicating that methylation is a major mechanism by which *Dkk-3* expression is silenced in digestive cancer cells. In addition, methylation of the *Dkk-3* promoter was observed frequently in primary gastric cancers (67.6%) and colorectal cancers (52.3%), leading to the conjecture that epigenetic silencing of the *Dkk-3* gene by promoter methylation is a common event in the tumorigenesis of gastric and colorectal cancers.

The functional relevance of the epigenetic silencing of *Dkk-3* determined was further in our cell culture experiments. We demonstrated that the constitutive expression of *Dkk-3* resulted in a significant decrease in colony formation. Inhibitory activity on cell growth by *Dkk-3* also has been reported in lung cancer cells,[17] osteosarcoma cells,[29] and digestive cancer cells.[21,26] Collectively, these observations provide strong evidence that the functional repression of *Dkk-3* caused by promoter methylation is involved in the biology of cancer and that *Dkk-3* may function as a suppressor of tumor growth.

At the time of diagnosis, most gastric and colorectal cancers already have reached an advanced tumor stage and metastasized to the lymph nodes. Consequently, the prognosis for these patients usually is poor. Therefore, it is still of paramount importance to identify tumor markers that predict patient prognosis more accurately than the conventional TNM tumor classification. Because of the high incidence of *Dkk-3* methylation in gastric cancer, we investigated the clinical significance and prognostic value of *Dkk-3* methylation in 104 patients with gastric cancers who had known survival data. Most noteworthy, *Dkk-3* methylation was correlated significantly with a poor prognosis in our study series and was independent of sex, age, *H. pylori* infection, Lauren type, and TNM tumor classification, suggesting that *Dkk-3* is an independent prognostic marker of prognosis for patients with gastric cancer. These results raise the possibility that aberrant methylation of *Dkk-3* has an important functional role in progression of gastric cancer. The potential value of *Dkk-3* methylation as a prognostic marker in colorectal cancer also was investigated in 84 patients who had colorectal cancers with known survival information. However, *Dkk-3* methylation was not associated with survival in colorectal cancer. This further emphasizes the important histologic differences in the prognostic importance of *Dkk-3* methylation in gastrointestinal cancers. Another observation that *Dkk-3* methylation predicts a poor patient outcome was made in lung adenocarcinomas.[30] Moreover, several previous groups have reported that the methylation of individual genes had prognostic significance, such as *CDH1* (E-cadherin) in diffuse gastric cancer,[31] death-associated protein (DAP) kinase (*DAPK*) in lung cancer,[32] sFRP-1 in papillary bladder cancer and breast cancer,[33,34] and the protein gene product 9.5/ubiquitin carboxyl-terminal esterase L1 gene *PGP9.5/UCHL1* in esophageal cancer.[35] Collectively, the results from our study suggest that *Dkk-3* methylation status has potential clinical use as a prognostic marker in patients with gastric cancer. The epigenetic silencing of the *Dkk-3* gene seems to characterize a more aggressiveness genotype of gastric cancer. Therefore, the specific restoration of *Dkk-3* expression also may be a promising novel treatment target for patients with gastric cancer and may improve their prognosis.

In summary, we observed that methylation of the *Dkk-3* promoter and the resultant gene silencing was a common event in gastric and colorectal cancers. In addition, we demonstrated that aberrant methylation of *Dkk-3* is an independent predictor of a poor prognosis in

patients with gastric cancer, indicating that these individuals may benefit from more aggressive treatment.

Conflict of Interest Disclosures

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