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Original Article

Methylation of the DKK3 promoter is associated with poor prognosis in patients with cervical adenocarcinoma

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Abstract: Objective: The aim of this study was to investigate the mRNA of DKK3 (Dickkopf-3) in cervical adenocarcinoma, and to explore correlations between methylation status of the DKK3 promoter and biological behaviors of cervical adenocarcinoma. Methods: The mRNA expression level of DKK3 was detected by real-time quantitative reverse transcription PCR. Methylation-specific PCR (MSP) analysis was performed to detect the methylated degrees of the DNA of the DKK3 promoter. Results: The mRNA expression levels of DKK3 in cervical adenocarcinoma tissues were lower than those in adjacent normal cervical tissues. MSP detection found DKK3 promoter methylation was 38% in cervical adenocarcinoma tissues, while no normal cervical tissues were found to be methylated. FIGO staging and pelvic lymph node metastasis were identified as relative factors of methylation status of the DKK3 promoter. Multivariate analysis demonstrated methylation status of the DKK3 promoter was an independent prognostic indicator of cervical adenocarcinoma. Patients with methylated DKK3 promoter exhibited significantly shorter OS than those with an unmethylated DKK3 promoter. Conclusions: The methylation status of the DKK3 promoter may indicate poor prognosis of patients with cervical adenocarcinoma.

Keywords: Cervical adenocarcinoma, DKK3, methylation, prognosis

Introduction

Cervical adenocarcinoma is one of the major causes of death in women with about 454,000 new cases and 200,000 deaths in 2010 worldwide [1]. Although diagnostic techniques and treatment methods have been improving, the prognosis of cervical adenocarcinoma is still dismal [2]. HPV infection, environmental, genetic, and epigenetic factors play important roles in cervical carcinogenesis. Epigenetics, defined as heritable changes in gene expression that are not coded in the DNA sequence itself, are increasingly linked with tumorigenesis [3]. Among epigenetic regulatory ways, histone methylation has demonstrated power of modifications over gene expression. It has been demonstrated that DNA methylation plays a key role in chromosomal stability, gene expression, genome imprinting, and transcriptional silencing of foreign DNA fragments [4]. Thus, exploring novel DNA methylation maybe needed to improve the prognosis of cervical adenocarcinoma.

DKK3 (Dickkopf-3) is a structurally and functionally divergent member of the DKK family of Wnt antagonists. DKK3 can modulate inflammatory cell activity, maintain tissue organization and can protect against myocardial infarction-induced fibrosis. DKK3 is also a tumour suppressor that inhibits proliferation of cancer cells and is downregulated in several types of human cancer [5-9]. Several reports have demonstrated that CpG island methylation of DKK3 results in low expression in caners [10]. However there are a few studies that have focused on the role of methylation status of the DKK3 promoter in cervical adenocarcinoma. In this study, we detected mRNA expression differences for DKK3 between cervical adenocarcinoma tissues and normal cervical tissues, and then determined the prognostic prediction value of DKK3 promoter methylation in patients with cervical adenocarcinoma.

Materials and methods

Ethics statement

This research was approved by the Ethics Committee of Tianjin Nankai Hospital and written informed consent was obtained from each patient involved in the study.

Patients and samples

Fresh cervical adenocarcinoma tissues and normal cervical tissues between June 2005 and December 2010 at Tianjin Nankai Hospital were collected to analyze mRNA and promoter methylation of DKK3. The tumour and normal cervical tissue samples were histologically verified. The inclusion criteria for this study included: (1) pathologically confirmed patients with FIGO IA-IIA cervical adenocarcinoma; (2) patients who underwent radical hysterectomy and bilateral pelvic LN dissection; (3) patients who had matched fresh surgical specimens and adjacent normal cervical tissues; (4) the clinical data and the follow-up information were complete. The exclusion criteria included: (1) patients who underwent palliative surgery; (2) patients who had distant metastasis or peritoneal dissemination that was confirmed during the operation; (3) patients who died during the initial hospital stay or within 1 month after surgery; and (4) patients who were lost to follow-up. Based on these inclusion and exclusion criteria, a total of 100 cervical adenocarcinoma patients were enrolled in this study.

DNA and RNA extraction

RNA was extracted from cervical adenocarcinoma tissues and normal cervical tissues by using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real-time quantitative PCR

Expression of GPRC5A mRNA was determined by quantitative real-time PCR (qRT-PCR) using ABI SYBR Green Master Mix (Life Technologies, CA, USA). Total sample RNA was normalized to endogenous GADPH mRNA. Primers designed and utilized for DKK3 and GAPDH were as follows: (forward/reverse sequences) DKK3 (5'-AGCTCTGGTCCTGGACTTCA/CAACCCACGACATGTAGCAC-3') and GAPDH (5'-TGGGTGTGAACCATGAGAAGT/TGAGTCCTTCCACGATACCAA-3'). Thermal cycler conditions included an initial hold period at 95°C for 2 min; this was followed by a two-step PCR program of 95°C for 10 s and 65°C for 40 s repeated for 40 cycles on an Stepone plus system (ABI, CA, USA). The relative quantification of GPRC5A expression was normalized to GAPDH value ($2^{-\Delta\Delta CT}$ method).

Methylation-specific PCR (MSP)

Cervical adenocarcinoma tissues and normal cervical tissues were subjected to qualitative methylation analysis of the DKK3 promoter by methylation-specific PCR (MSP). The following DKK3 primers were used to detect the methylated (M) or unmethylated (U) alleles of the DKK3 promoter: for methylated alleles, DKK3 forward (5'-CGGTTTTTTTTCGTTTTCGGG-3'), and reverse (5'-CAAACCGCTACATCTCCGCT-3'); for unmethylated alleles, DKK3 forward (5'-TTTTGGTTTTTTTTTGTTTTTGGG-3'), and reverse (5'-CCAAACCACTACATCTCCACT-3'). A total of 25 cycles of MSP were performed using Ampli Taq-Gold (methylation-specific primers, annealing temperature 60°C; unmethylation-specific primers, annealing temperature 58°C). MSP primers were initially evaluated to verify whether or not any unbisulphited DNA was amplified, and the specificity of MSP was further confirmed by directly sequencing some PCR products. PCR was resolved using 2% agarose gel. Image J software was used to calculate the relative values of the methylation of the DKK3 promoter in the 100 cervical adenocarcinoma tissues. Hypermethylation of the DKK3 promoter was defined from the calculated methylation value of cervical adenocarcinoma tissue not less than the positive control value.

Statistical analysis

Associations between gene expression and clinicopathological factors were analyzed using chi-square test. Kaplan-Meier method and log-rank tests were employed to correlate gene expression levels and patient survival in univariate analysis. Multivariate Cox-regression analyses were conducted to identify independent prognostic factors. Significance was set at $P< 0.05$. Statistical calculations were performed using SPSS Statistics 17.

Result

Patient characteristics

The clinicopathological characteristics of the patients are shown in **Table 1**. Among these

Table 1. Clinicopathological characteristics of patients with cervical adenocarcinoma

Variables	Cases	Mean ± SD
Age (years)		21-79
≤45	66	
>45	34	
Size of tumor (cm)		0.5-10
≤4	68	
>4	32	
FIGO staging		
IA	35	
IB	25	
IIA	40	
Tumor grade		
Well/Moderate	51	
Poor	49	
Deep cervical stromal invasion		
Yes	37	
No	63	
Pelvic lymph node metastasis		
Yes	69	
No	31	
Neoadjuvant chemotherapy		
Yes	31	
No	69	

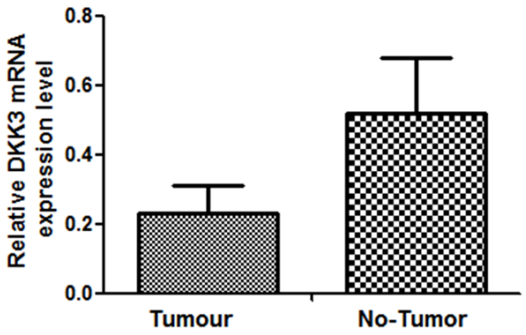

Figure 1. Relative DKK3 mRNA expression in cervical adenocarcinoma tissues and normal cervical tissues assessed by real-time qRT-PCR (P<0.001).

patients, 31 cases received 2-3 cycles of chemotherapy before operation. The chemotherapy regimens were paclitaxel at 175 mg/m^2 on day 1, cisplatin at 60 mg/m^2 on day 1; repeated every 3 weeks.

DKK3 mRNA expression levels

The mRNA levels of DKK3 were estimated by qRT-PCR assays on 20 of 100 cervical adenocarcinoma tissues and the 20 normal cervical tissues. The relative mRNA expression values of DKK3 in cervical adenocarcinoma tissues were lower than those in normal cervical tissues (0.23±0.08 vs 0.52±0.16, P<0.001) (**Figure 1**).

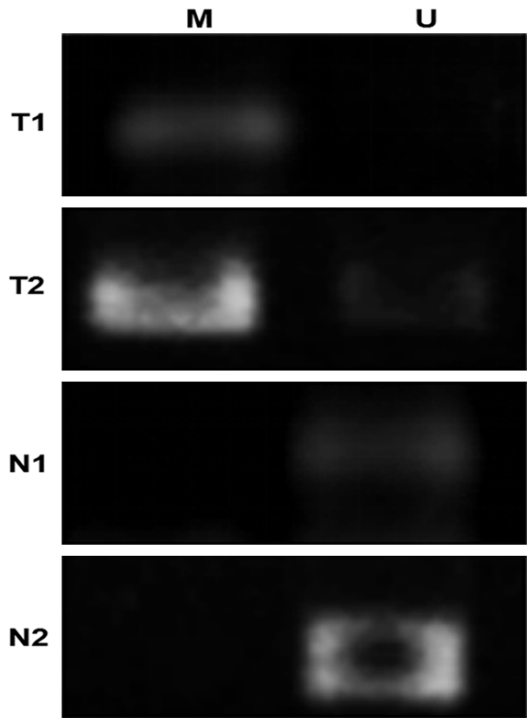

Figure 2. MSP detection of DKK3 promoter methylation in different cervical adenocarcinoma tissues and normal cervical tissues. (Representation: T, cervical adenocarcinoma tissues; N, normal cervical tissues; M, methylated; U, unmethylated).

Methylation of the DKK3 promoter

MSP analysis found the methylation of the DKK3 promoter in 100 cervical adenocarcinoma tissues; while the DKK3 promoter was not methylated in the 20 normal cervical tissues (**Figure 2**). Among 100 cervical adenocarcinoma tissues, 38 presented methylated DKK3 promoter, while 62 presented with unmethylated DKK3 promoter.

Relationships between methylation status of the DKK3 promoter in cervical adenocarcinoma tissues and various clinicopathological characteristics

FIGO staging (P = 0.016) and pelvic lymph node metastasis (P = 0.033) were identified as relative factors of methylation status of the DKK3 promoter in cervical adenocarcinoma tissues (**Table 2**).

Table 2. Relationships between methylated status of DKK3 promoter and various clinicopathological variables in patients with cervical adenocarcinoma

Variables	Methylated status of DKK3 promoter		*P* value
	Methylated	Unmethylated	
Age (years)			0.088
≤45	29	37	
>45	9	25	
Size of tumor (cm)			0.090
≤4	22	46	
>4	16	16	
FIGO staging			0.016
IA	10	25	
IB	6	19	
IIA	22	18	
Tumor grade			0.071
Well/Moderate	15	36	
Poor	23	26	
Deep cervical stromal invasion			0.668
Yes	15	22	
No	23	40	
Pelvic lymph node metastasis			0.033
Yes	31	38	
No	7	24	
Neoadjuvant chemotherapy			0.151
Yes	15	16	
No	23	46	

Survival analysis of patients with cervical adenocarcinoma

Univariate analysis showed significant relationships between the OS and FIGO staging, tumor grade, pelvic lymph node metastasis, and methylation status of the DKK3 promoter ($P<0.05$); by contrast, no significant relationships were detected between OS and age, size of tumor, deep cervical stromal invasion, and neoadjuvant chemotherapy ($P>0.05$). Patients with methylated DKK3 promoter exhibited significantly shorter OS than those with unmethylated DKK3 promoter (**Table 3**).

Furthermore, multivariate survival analysis showed FIGO staging (HR = 2.047; $P = 0.001$), tumor grade (HR = 3.699; $P<0.001$), pelvic lymph node metastasis (HR = 2.100; $P = 0.036$) and methylated status of DKK3 promoter (HR = 0.568; $P = 0.039$) as independent predictors of OS of patients with cervical adenocarcinoma (**Figure 3**).

Discussion

The Dickkopf family of secreted glycoproteins is composed of four members that in early metazoans are key regulators of the Wnt/β-catenin signaling pathway [11]. Three family members DKK1, DKK2, and DKK4 bind to the LRP5/6 and Kremen subunits of the receptor and prevent assembly of a functional Wnt receptor complex [12]. While DKK3 retains the two cysteine rich domains common to all family members despite its inability to disrupt Wnt receptor binding, DKK3 is the best-known tumor suppressor in the family [13]. Much evidence exists to identify REIC/DKK3 as a tumor suppressor and confirm its differential expression in many solid tumors; DKK3 affects tumor cell growth due to induction of apoptosis in mammary carcinomas and several other cancer types and might be involved in the inhibition of epithelial-to-mesenchymal transition (EMT) [14]. DKK down regulation has been reported in endometrial cancer, lung cancer, gastrointestinal cancer, breast cancer, prostate cancer, and renal carcinomas [15]. Nozaki et al. [16] found reduced expression in 63% of lung cancer tissues compared to match adjacent normal tissues. Some studies found aberrant methylation of DKK3 gene was detected in 32.1% cervical adenocarcinoma tumors. Another study showed DKK3 as a tumor suppressor in human pancreatic cancer, which plays roles through the downregulation of β-catenin expression via the ERK-mediated pathway [17]. In cervical cancers, DKK3 was found to be frequently downregulated when compared to normal cervical tissue. In contrast, others have reported that serum DKK3 was increased in both endometrial and cervical cancer patients when compared to healthy people [18].

The promoter region of the DKK3 gene contains CpG islands, and downregulation of DKK3 in cancer correlates with methylation on the CpG islands [19]. DNA methylation commonly refers to the covalent addition of a methyl (-CH3) group from the s-adenosylmethionine (SAM) to the fifth carbon of the cytosine base (5 mC) which is catalyzed by DNA methyltransferases (DNMTs) enzymes [20]. Loss of DKK3 expression is mainly mediated by promoter hypermethylation as demonstrated in the majority of tumor entities [21]. Lorsy E et al. [22] found that the Wnt antagonist gene DKK3 showed a very similar frequency of promoter methylation in human breast cancer, and DKK3

Table 3. Survival analysis of cervical adenocarcinoma patients

Variables	Univariate analysis		Multivariate analysis	
	χ^2 value	*P* value	HR value	*P* value
Age (years)	0.913	0.399		
≤45				
>45				
Size of tumor (cm)	0.864	0.353		
≤4				
>4				
FIGO staging	45.457	<0.001	2.047	0.001
IA				
IB				
IIA				
Tumor grade	41.678	<0.001	3.699	<0.001
Well/Moderate				
Poor				
Deep cervical stromal invasion	2.424	0.119		
Yes				
No				
Pelvic lymph node metastasis	15.299	<0.001	2.100	0.036
Yes				
No				
Neoadjuvant chemotherapy	0.913	0.339		
Yes				
No				
DKK3 promoter methylation status	13.896	<0.001	0.568	0.039
Methylation				
Unmethylation				

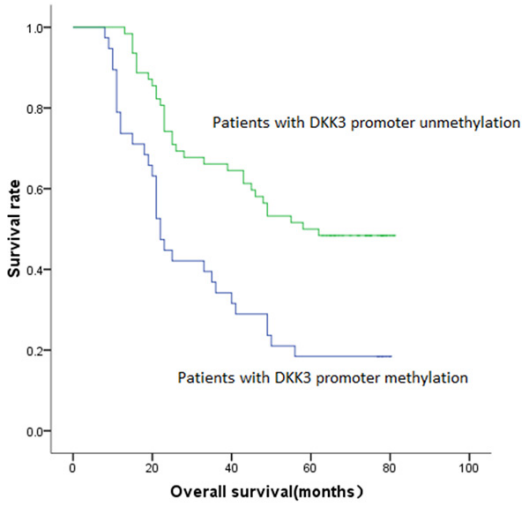

Figure 3. Survival curve of 100 patients with cervical adenocarcinoma according to methylation status of the DKK3 promoter (methylated or unmethylated).

methylation proved to be a novel prognostic marker potentially useful in the clinical management of this disease. Other studies found methylation of DKK3 was an important event in early malignant transformation and HCC progression, and therefore might be a prognostic indicator for risk assessment of HCC [23]. However there were a few studies focused on role of methylated status of DKK3 promoter in cervical adenocarcinoma. In this study, we investigated DKK3 expression in cervical adenocarcinoma and its correlation with clinicopathological characteristics of patients, including OS.

We first investigated DKK3 mRNA expression in cervical adenocarcinoma specimens by qRT-PCR, and found that mRNA expression levels of DKK3 in cervical adenocarcinoma tissues were both significantly lower than those in normal cervical tissues. In addition, we found that 38% of cervical adenocarcinoma tissues presented methylated DKK3 promoter, while none of normal cervical tissues presented with methylated DKK3 promoter. Therefore, these findings indicate that DKK3 may function as a tumor suppressor gene in cervical adenocarcinoma and may be tested as an epigenetic biomarker to diagnose cancer.

Besides the results mentioned above, we found that FIGO staging and pelvic lymph node metastasis were independent relative factors of methylation status of the DKK3 promoter. These results may indicate that methylation status of the DKK3 promoter may affect invasion, metastasis, and progression of cervical adenocarcinoma.

To assess the prognostic value of methylation status of the DKK3 promoter in cervical adenocarcinoma, our results showed that FIGO staging, tumor grade, pelvic lymph node metastasis, and methylated status of DKK3 promoter

as independent factors of OS. Furthermore we found that patients with a methylated DKK3 promoter exhibited significantly shorter OS than those with an unmethylated DKK3 promoter. This result indicates that the methylation status of the DKK3 promoter is an optimal predictor of cervical adenocarcinoma patients' prognosis. Thus, our study demonstrates that methylation of the DKK3 promoter might be an important prognostic biomarker for patients with cervical adenocarcinoma.

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Disclosure of conflict of interest

None.

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