***DKK3* is a potential tumor suppressor gene in papillary thyroid carcinoma**

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Abstract

The expression of the Dickkopf homolog 3 (*DKK3*) gene is downregulated in some human cancers, suggesting a possible tumor suppressor role of this gene. The role and regulation of *DKK3* in thyroid cancer have not been examined. In this study, we explored the relationship of promoter methylation with the inactivation of *DKK3* and tumor behaviors in papillary thyroid carcinoma (PTC). We used methylation-specific PCR and RT-PCR to examine the promoter methylation and expression of *DKK3* and tumor characteristics. We found mRNA expression of *DKK3* in 44.9% of the PTC tissue samples vs 100% of the matched normal thyroid tissue samples ($P < 0.01$). In contrast, an opposite distribution pattern of *DKK3* gene methylation was observed; specifically, 38.8% of the PTC tissue samples vs 0% of the matched normal thyroid tissue samples harbored *DKK3* methylation. An inverse correlation between the promoter methylation and mRNA expression of *DKK3* in PTC tissue samples was also observed. Moreover, we also found an inverse correlation between *DKK3* expression and some aggressive pathological characteristics of PTC, including high TNM stages and lymph node metastasis, but a positive correlation between *DKK3* promoter hypermethylation and pathological aggressiveness of the tumor. Treatment of the PTC cell line TPC-1 with the demethylating agent 5-azaC reduced *DKK3* promoter methylation and enhanced its expression, establishing functionally the impact of *DKK3* methylation on its expression. Our data thus for the first time demonstrate that the *DKK3* gene is a potential tumor suppressor gene in thyroid cancer and that aberrant promoter methylation is an important mechanism for its downregulation, which may play a role in the tumorigenesis and aggressiveness of PTC.

Key Words

- ▶ thyroid carcinoma
- ▶ *DKK3*
- ▶ DNA methylation
- ▶ tumor suppressor gene
- ▶ thyroid tumorigenesis

Endocrine-Related Cancer
(2013) 20, 507–514

Introduction

Thyroid carcinoma is the most common endocrine malignancy, and papillary thyroid carcinoma (PTC) accounts for over 90% of all thyroid malignancies

(Yin et al. 2010a). The incidence of thyroid cancer, mainly PTC, has been rising rapidly in recent decades worldwide (Davies & Welch 2006). It has been well established that

both genetic and epigenetic alterations contribute to carcinogenesis (Yin et al. 2011). In particular, hypermethylation of CpG islands in the promoter regions of tumor suppressor genes (TSGs) is commonly observed in various cancers, including thyroid cancer (Xing 2007). DNA methylation is a common epigenetic event observed in mammalian genomes, and it often mediates transcriptional repression (Alegría-Torres et al. 2011). It has been reported that many TSGs are hypermethylated in PTC (Lal et al. 2006, Xing 2007, Yin et al. 2010b, Stephen et al. 2011).

The Dickkopf (DKK) family consists of four members that share two conserved cysteine-rich domains (Fong et al. 2009). It has been demonstrated that DKKs, including DKK3, display regionalized expression and control cell fate during development in vertebrates (Monaghan et al. 1999, Suwa et al. 2003, Ang et al. 2004, Diep et al. 2004, Fjeld et al. 2005, Nie 2005). DKK1, DKK2, and DKK4, antagonists of Wnt signaling (Krupnik et al. 1999, Veeck & Dahl 2012), interact with Wnt coreceptors, LDL receptor-related protein 5/6 (LRP5/6) and kremen proteins (Davidson et al. 2002, Mao et al. 2002). DKK3 interacts with kremen1 and 2, but not with LRP5/6 (Nakamura & Hackam 2010). Among the DKK family members, REIC/DKK3 is rather unique with regard to the homology analysis of the DNA sequence, expression profiles, and biological functions (Glinka et al. 1998, Krupnik et al. 1999). The DKK3 protein is ubiquitously expressed in mouse and normal human tissues, but its expression is significantly downregulated in several cancers (Abarzua et al. 2005, Zhang et al. 2010). However, the expression and role of *DKK3* in thyroid cancer have not been examined. To better understand the role of *DKK3* in PTC, we investigated and compared *DKK3* expression and promoter methylation in PTC tissues and their adjacent normal tissues. Our data demonstrated that *DKK3* expression is markedly reduced in primary PTC tissues than in normal thyroid tissues. We also provided evidence that the loss of *DKK3* expression in PTC is predominantly mediated by the hypermethylation of the *DKK3* promoter, thus identifying *DKK3* as a potentially important TSG in PTC.

Materials and methods

Materials

Thyroid tumor and adjacent normal thyroid tissue samples were obtained from 49 thyroid carcinoma patients during the primary thyroid surgery at The First Affiliated Hospital of Zhengzhou University under

an Institutional Review Board-approved protocol at Zhengzhou University, between October 2010 and April 2011. After collection, tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C .

The patients comprised 18 males and 31 females with ages ranging between 15 and 75 years (median age, 45 years). The patients had not undergone prior chemotherapy or radiotherapy. The clinicopathological staging of PTC was made based on the TNM classification system introduced in 2002 by the American Joint Committee on Cancer (Greene et al. 2002; New York, Springer-Verlag). Among the patients, 19 had grade I and 30 had grade II carcinomas according to histological grading. By clinical staging using TNM standards, 4 patients had stage I, 18 had stage II, 26 had stage III, and 1 had stage IV carcinomas. The presence of lymphatic metastasis in regional nodes was confirmed pathologically and was positive in 28 patients.

Cell culture

TPC-1 cell line was kindly provided by Dr Ye Lei (Shanghai Rui Jin Hospital). TPC-1 cells were cultured and maintained in RPMI-1640 medium with 10% fetal bovine serum according to ATCC propagation guidelines in a humidified incubator with 5% CO_2 at 37°C .

RT-PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen) as described by the manufacturer. One microgram of total RNA was reverse transcribed into cDNA using the Reverse Transcriptase and oligo (dT; Fermentas, Burlington, Ontario, Canada). To detect *DKK3* by PCR, the sense primer, 5'-ACAGCCACAGCCTGGTGTA-3' and antisense primer, 5'-CCTCCATGAAGCTGCCAAC-3' were used (Veeck et al. 2008), resulting in a PCR product of 120 bp. *GAPDH* was also detected as a control, using the following primers: sense primer, 5'-GACCACAGTC-CATGCCATCAC-3' and antisense primer, 5'-GTCCAC-CACCCTGTTGCTGTA-3'. The length of amplified *GAPDH* was 454 bp. PCR consisted of an initial denaturation step at 95°C for 30 s, followed by 29 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min and 4°C to stop the reaction. The resulting PCR product was resolved by 2% agarose gel electrophoresis and stained with ethidium bromide. The intensities of the *DKK3* band were analyzed using the Quantity One Software and normalized to those of the *GAPDH* band. The results obtained from the tumor

Figure 1

(A) Representative images of *DKK3* mRNA expression in PTC tissues and the adjacent non-cancerous epithelial tissues, detected using RT-PCR. (B) Positive rate of *DKK3* mRNA expression in 49 PTC tissue samples and their adjacent non-cancerous epithelial tissue samples. SM, Standard Marker DL-500; C, cancer tissue of PTC; N, matched adjacent non-cancerous epithelial tissues; PTC, papillary thyroid cancer; NCE, non-cancerous epithelial tissues.

tissue samples were compared with those obtained from the matched normal tissue samples (average). A result greater than this value or in this value range was considered positive gene expression. A result below this value or absent was considered low or absent expression (for statistical analyses, both were considered impaired/negative gene expression).

Methylation-specific PCR

To analyze the methylation status of the promoter of the *DKK3* gene, we used previously reported primers (Veeck et al. 2008). Extraction of genomic DNA (gDNA) was carried out using a gDNA extraction kit (BioTeke, Beijing, China). The snap-frozen samples were dissolved in the lysis buffer followed by DNA isolation. Bisulfite-modified gDNA was prepared using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to manufacturer's instructions. For the bisulfite reaction, 400 ng gDNA and 130 μ l CT conversion reagent were used. Sample tubes were placed in a thermal cycler (MJ Research, Waltham, MA, USA), and the following steps were carried out: 10 min at 98 $^{\circ}$ C and 2.5 h at 64 $^{\circ}$ C, and then stored at 4 $^{\circ}$ C. The resultant DNA was purified using the EZ DNA Methylation-Gold kit. DNA samples were finally stored at -20 $^{\circ}$ C for further study. PCR was performed using a volume of 50 μ l. Amplification was carried out according to the general

Table 1 Relationship between *DKK3* mRNA expression and clinicopathological features in PTC.

Clinical data	n	Expression of mRNA		Positive rate (%)	χ^2	P
		Positive	Negative			
Gender						
Male	18	6	12	33.3	1.538	0.215
Female	31	16	15	51.6		
Age (years)						
<45	23	10	13	43.5	0.035	0.851
≥ 45	26	12	14	46.2		
Tumor size (cm)						
<2	24	9	15	37.5	1.041	0.308
≥ 2	25	13	12	52.0		
TNM stage						
I-II	22	15	7	68.2	8.749	0.003*
III-IV	27	7	20	26.0		
Tumor pathological grade						
I	19	12	7	63.2	4.182	0.041 [†]
II	30	10	20	33.3		
Lymph node metastasis						
No	21	13	8	62.0	4.296	0.038 [†]
Yes	28	9	19	32.1		

* $P < 0.01$ and $^{\dagger}P < 0.05$.

guidelines suggested for pyrosequencing (TakaRa Taq Hot Start Version; Takara, Dalian, Shandong, China): denaturation at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 60 °C for 30 s for unmethylated primers and 58 °C for 30 s for methylated primers, 72 °C for 45 s, and the final extension at 72 °C for 5 min and 4 °C to stop the reaction. The PCR product (5 µl) was confirmed by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining.

Demethylation

TPC-1 cells were treated with 5-azaC at various concentrations for 48 h. Methylation-specific PCR (MSP) analysis was conducted to evaluate the promoter methylation status of the *DKK3* gene in the TPC-1 cells, and RT-PCR was used to determine the mRNA expression of the *DKK3* gene. The sense primer, 5'-GAGTCAACGGATTGTCGT-3' and antisense primer, 5'-GACAAGCTTCCCGTTGTCAG-3' were used to amplify *GAPDH* (185 bp). The primers and process for MSP and RT-PCR were as those stated above.

Statistical analyses

Statistical analyses were performed using SPSS Software version 10.0. Significance was analyzed using the χ^2 -, *t*-test, and one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

DKK3 mRNA expression levels in primary tumors

DKK3 mRNA expression in tumor tissues and their adjacent normal tissues was analyzed using RT-PCR assay (Fig. 1). We were able to detect *DKK3* mRNA expression in all the 49 non-cancerous epithelial (NCE) tissue samples, while only 22 of the 49 (44.9%) PTC tissue samples displayed positive *DKK3* mRNA expression, indicating that *DKK3* transcriptional repression occurred in at least half of the PTC tissue samples. Further analysis indicated that the mRNA expression of the *DKK3* gene was negatively correlated with the tumor pathological grade, TNM stage, and lymph node metastasis ($P < 0.05$; Table 1), suggesting a role of the inactivation of *DKK3* in the progression of PTC.

Methylation of the *DKK3* gene in primary tumors

No *DKK3* promoter methylation was observed in the 49 NCE tissue samples, while 19 of the 49 (38.8%) PTC tissue

Figure 2

(A) Representative images of *DKK3* promoter methylation in PTC tissues and the adjacent non-cancerous epithelial tissues, detected using methylation-specific PCR. (B) Rate of *DKK3* promoter hypermethylation in 49 PTC tissue samples and their adjacent non-cancerous epithelial tissue samples. SM, Standard Marker DL-500; C, cancer tissue; N, non-cancerous epithelial tissues; U, unmethylated; M, methylated; PTC, papillary thyroid cancer; NCE, non-cancerous epithelial tissues.

samples exhibited hypermethylation in the *DKK3* promoter (Fig. 2). This result indicates that the low expression or abrogation of *DKK3* mRNA in PTC might be attributed to the hypermethylation of the *DKK3* promoter. The χ^2 analysis suggested that the promoter hypermethylation of the *DKK3* gene was positively related to the TNM stage, tumor pathological grade, and lymph node metastasis ($P < 0.05$) in PTC (Table 2).

Relationship between the promoter methylation and mRNA expression of *DKK3* in PTC

We further analyzed whether promoter methylation is associated with the downregulation of *DKK3* expression in PTC. Among 19 cases with *DKK3* promoter hypermethylation, 15 exhibited negative mRNA expression, and the χ^2 analysis indicated a negative correlation between the promoter methylation and mRNA expression of *DKK3* ($P < 0.05$; Table 3).

Table 2 Relationship between *DKK3* promoter hypermethylation and clinicopathological features in PTC.

Clinical data	n	Methylation of <i>DKK3</i>		Positive rate (%)	χ^2	P
		Methylated	Unmethylated			
Gender						
Male	18	8	10	44.4	0.385	0.535
Female	31	11	20	35.5		
Age (years)						
<45	23	12	11	52.2	3.278	0.07
≥45	26	7	19	27.0		
Tumor size (cm)						
<2	24	12	12	50.0	2.496	0.114
≥2	25	7	18	28.0		
TNM stage						
I–II	22	5	17	22.8	4.331	0.037*
III–IV	27	14	13	51.9		
Tumor pathological grade						
I	19	4	15	21.1	4.106	0.043*
II	30	15	15	50.0		
Lymph node metastasis						
No	21	4	17	19.0	6.025	0.014*
Yes	28	15	13	53.6		

* $P < 0.05$.**Effect of a demethylating agent on *DKK3* gene expression**

To examine the relationship between the expression and methylation of *DKK3*, we analyzed the effect of the demethylating agent 5-azaC on the promoter methylation and mRNA expression of the *DKK3* gene in TPC-1 cells. As shown in Figs 3 and 4, after treatment of the TPC-1 cells with 5, 10, 20, and 50 $\mu\text{mol/l}$ of 5-azaC for 48 h, the relative expression levels of *DKK3* mRNA were 0.208 ± 0.017 , 0.365 ± 0.013 , 0.489 ± 0.017 , and 0.582 ± 0.011 respectively (Fig. 3). Meanwhile, the *DKK3* gene was demethylated partially after treatment with 5-azaC ($F_m = 315.188$, $F_{um} = 195.257$; $P < 0.05$; Fig. 4). These data indicate that treatment with 5-azaC significantly enhanced *DKK3* mRNA expression, while it reduced *DKK3* promoter methylation in the TPC-1 cells, suggesting that *DKK3* is indeed epigenetically silenced in TPC-1 cell line.

Discussion

Epigenetic alterations, such as DNA hypermethylation, play a critical role in tumorigenesis (Schuebel et al. 2007, Ushijima & Asada 2010). Over the past several years, many TSGs have been found to be epigenetically inactivated in PTC, indicating that epigenetic silencing of TSGs is a major molecular alteration in the tumorigenesis of thyroid carcinoma (Lal et al. 2006, Xing 2007, Yin et al.

2010b, Stephen et al. 2011). It has recently been shown that the methylation of *DKK3* gene family contributes to carcinogenesis and serves as a potential biomarker for the diagnosis of several human malignancies (Veeck et al. 2008, 2009, Vander Meide et al. 2011). *DKK3* is expressed in many normal human tissues (Hermann et al. 2007, Du et al. 2011), and its expression is downregulated in several human cancers such as lung cancer (Tsuji et al. 2001, Kobayashi et al. 2002, Licchesi et al. 2008, Yue et al. 2008), pancreatic cancer (Hsieh et al. 2004), bladder cancer (Urakami et al. 2006), melanoma (Kuphal et al. 2006), and gastrointestinal tumors (Sato et al. 2007). The role of the *DKK3* gene and its epigenetic alterations remain unknown in thyroid cancer.

In this study, we identified *DKK3* as another potential TSG whose epigenetic inactivation might contribute to

Table 3 Relationship between the promoter methylation and mRNA expression of *DKK3*.

Promoter methylation	mRNA expression		Total
	+	–	
+	4	15	19
–	18	12	30
Total	22	27	49

 $\chi^2 = 7.132$, $P = 0.008$.

Figure 3

(A) Increase in the expression of *DKK3* mRNA detected by RT-PCR in response to the treatment with 5-azaC in a concentration-dependent manner. (B) Bar graph showing the concentration-dependent effects of 5-azaC on the expression of *DKK3*, corresponding to (A). *GAPDH* was used as a quantitative control. SM, Standard Marker DL-500. Unlike that mentioned in Figs 1 and 2 SM in this figure was obtained from a different company that had no MW marker with 350 bp.

the tumorigenesis of PTC. One of the strengths of this study is that both the expression and methylation status of the *DKK3* promoter were analyzed in a large number of cases of PTC and their matched NCE tissue samples. The positive rate of *DKK3* gene expression in the PTC tissues was significantly lower than that in the NCE tissues ($P < 0.01$). Similar to our study, Kurose *et al.* (2004) examined 17 pairs of human renal clear-cell carcinoma tissues and their adjacent normal tissues and demonstrated that *DKK3* mRNA levels were significantly decreased in carcinoma tissues. In the 49 NCE tissue samples that we examined, no promoter hypermethylation of the *DKK3* gene was found, whereas *DKK3* promoter methylation was observed in 38.8% of the cases of PTC. Lodygin *et al.* (2005) found that abnormal methylation of the *DKK3* gene was present in 28 of 41 (68%) cases of prostate cancer, but absent in normal controls. We also found that the expression of *DKK3*

mRNA was lower while the incidence of *DKK3* promoter methylation was higher in the cases of PTC that had advanced disease stages or metastasis compared with those that had early disease stages and no metastasis. In addition, we also observed that tumors in which methylation of the *DKK3* promoter region was absent had high levels of expression. Using the PTC cell line TPC-1 and a demethylating agent, we were able to functionally link the promoter methylation of *DKK3* to decreased *DKK3* expression, establishing the promoter methylation of *DKK3* as an important mechanism for its inactivation in PTC. Similarly, Roman-Gomez *et al.* (2004) analyzed CpG island methylation of the *DKK3* promoter in six ALL cell lines and found that *DKK3* promoter hypermethylation existed in all of them and was associated with decreased *DKK3* mRNA expression, which could be restored after exposure to the demethylating agent 5-azaC. These findings together with ours indicate that the promoter of the *DKK3* gene is commonly methylated and silenced in human cancer cells, consistent with its role as a potential TSG. TSG methylation can be associated with *BRAF* mutation (Hu *et al.* 2006, Guan *et al.* 2008), and the later

Figure 4

(A) Partial demethylation of the *DKK3* gene promoter in TPC-1 cells by treatment with 5-azaC. (B) Bar graph of the results obtained for *DKK3* gene demethylation after treatment with 5-azaC, corresponding to (A). SM, Standard Marker DL-500; U, unmethylated; M, methylated. Unlike that mentioned in Figs 1 and 2, SM in this figure was obtained from a different company that had no MW marker with 350 bp. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-13-0053>.

is associated with the development of tumor-promoting microenvironments (Nucera *et al.* 2010). It would be interesting to investigate whether this is the case with the *DKK3* gene in a larger study in the future.

Another important finding of this study is that the promoter methylation and decreased expression of the *DKK3* gene were associated with aggressive clinico-pathological characteristics of PTC, suggesting that the epigenetic inactivation of *DKK3* may play an important role in thyroid tumorigenesis and progression and that *DKK3* is a major thyroid TSG. As such the hypermethylation of *DKK3* may have a prognostic value in the risk stratification of PTC.

In summary, our results suggest that *DKK3* is a potential new TSG in PTC. *DKK3* may play an important role in the tumorigenesis and aggressiveness of PTC, and the methylation or expression status of *DKK3* may be a potentially useful biomarker for evaluating the biological behavior and prognosis of PTC. These results may also bear therapeutic implications for PTC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Henan Medical Science and Technology Project Fund (201201004 D Yin) and Youth Innovation Fund of the First Affiliated Hospital of Zhengzhou University (2011117 D Yin).

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Received in final form 15 May 2013

Accepted 23 May 2013

Made available online as an Accepted Preprint

23 May 2013