Hypermethylation of *CDH13*, *DKK3* and *FOXL2* promoters and the expression of EZH2 in ovary granulosa cell tumors

YANMEI XU[1,2], XIA LI[3], HONGTAO WANG[4], PENGMU XIE[2], XUN YAN[2], YU BAI[2] and TINGGUO ZHANG[1]

[1]Institute of Pathology and Pathophysiology, Shandong University School of Medicine, Jinan, Shandong 250012;
[2]Department of Pathology, Shandong Shanxian Central Hospital, Heze, Shandong 274300;
[3]Department of Pathology, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032;
[4]Department of Gynecology and Obsterics, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research for Cancer, Tianjin 300060, P.R. China

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Abstract. Aberrant epigenetic modification is associated with the development and progression of cancer. Hypermethylation of tumor suppressor gene promoters and cooperative histone modification have been considered to be the primary mechanisms of epigenetic modification. Ovary granulosa cell tumors (GCTs) are relatively rare, accounting for ~3% of all ovarian malignancies. The present study assessed hypermethylation of the cadherin 13 (*CDH13*), dickkopf WNT signaling pathway inhibitor 3 *(DKK3)* and forkhead box L2 (*FOXL2*) promoters in 30 GCT tissues and 30 healthy control tissues using methylation-specific polymerase chain reaction analysis. The data showed that the frequencies of *CDH13*, *DKK3* and *FOXL2* promoter methylation were significantly higher in the GCT tissues, compared with the healthy control tissues (86.67, vs. 23.33%; 80, vs. 26.67% and 66.67, vs. 20%, respectively; P<0.001). Immunostaining of enhancer of zeste homolog 2 (EZH2), a histone H3K27 methyltransferase, showed that the EZH2 protein was expressed in 11 of the 30 GCT tissue samples, whereas no EZH2 protein was expressed in the 30 healthy control tissues (P<0.01). These data suggested that hypermethylation of the *CDH13*, *DKK3* and *FOXL2* gene promoters, and overexpression of the EZH2 protein were involved in the development of GCT.

Introduction

Granulosa cell tumors (GCTs) are malignant neoplasms, which originate from the sex cord. GCT is a relatively rare disease, but commonly occurs in the ovary. GCTs frequently occur in young women, and the majority of GCT cases are diagnosed in the late stages due to early stage GCT exhibiting few clinical symptoms, leading to a poor prognosis (1,2). Due to the current lack of understanding of this particular disease, the clinical management of GCTs is similar to that of other types of epithelial ovarian cancer. However, unlike other types of ovarian cancer, the prognostic assessment for GCT predominantly relies on clinicopathological variables, including stage and grade, although these do not provide biological insight into the disease (3). Thus, early diagnosis is critical for the successful management of numerous types of human cancer, including ovarian GCTs.

The identification of molecular or epigenetic markers may provide biological insight into GCT and a serve as a critical tool in successful treatment of the disease. Current knowledge indicates that the development and progression of cancer are driven by the accumulation of genetic abnormalities and epigenetic alterations, which include gene mutations and silencing, and epigenetic modifications of genomic DNA, including methylation of DNA CpG islands or covalent modification of histone tails (4,5). Gene promoter methylation silences the expression of tumor suppressor genes, which is one of the key mechanisms in tumor development (6,7). Aberrant gene methylation is also one of the earliest molecular alterations occurring in tumorigenesis, and is considered a biomarker for early tumor detection or a potential treatment strategy (8-10). Histone modification also regulates genetic programs in normal cells, but is altered in cancer cells (11). Therefore, the pathogenesis of GCT may follow a similar trend to other types of human cancer.

In the present study, the methylation status of three putative tumor suppressor genes, cadherin 13 (*CDH13*), dickkopf WNT signaling pathway inhibitor 3 *(DKK3)* and forkhead box L2 (*FOXL2*), and expression of enhancer of zeste homolog 2 (EZH2) were assessed in GCT tissue samples and compared with follicular cyst tissues. The aim of the present study was to screen and identify tumor markers for the early detection of GCT. As GCTs are rare and reports are limited, the present study focused on *CDH13, DKK3* and *FOXL2*, as these are putative tumor suppressor genes, whose functions and expression are associated with the ovary, including ovary development,

Correspondence to: Dr Tingguo Zhang, Institute of Pathology and Pathophysiology, Shandong University School of Medicine, 44 Wenhua Xi Road, Jinan, Shandong 250012, P.R. China
E-mail: xymhky@126.com

Key words: granulosa cell tumors, DNA methylation, histone modifications, tumor suppressor gene, clinicopathological features

Table I. Primer sequences for PCR amplification of multiple tumor suppressor genes and methylation-specific PCR analysis of gene promoter methylation.

Gene	Sequence	Amplicon (bp)	Temperature (˚C)
CDH13-M-F	5'-TCGCGGGGTTCGTTTTTCGC-3'	243	58
CDH13-M-R	5'-GACGTTTTCATTCATACACGCG-3'		
CDH13-U-F	5'-TTGTGGGGTTTGTTTTTTGT-3'	242	
CDH13-U-R	5'-AACTTTTCATTCATACACACA-3'		
DKK3-M-F	5'-GGGGCGGGCGGCGGGGC-3'	120	60
DKK3-M-R	5'-ACATCTCCGCTCTACGCCCG-3'		
DKK3-U-F	5'-TTAGGGGTGGGTGGTGGGGT-3'	126	
DKK3-U-R	5'-CTACATCTCCACTCTACACCCA-3'		
FOXL2-M-F	5'-GTTATAATATTTTTTCGGTTGTTC G-3'	211	58
FOXL2-M-R	5'-CTAACTCCACGACCTATACTCGAT-3'		
FOXL2-U-F	5'-AGGTTATAATATTTTTTTGGTTGTTTG-3'	214	
FOXL2-U-R	5'-CCTAACTCCACAACCTATACTCAAT-3'		

PCR, polymerase chain reaction; CDH13, cadherin 13; DKK3, dickkopf WNT signaling pathway inhibitor 3; FOXL2, forkhead box L2; M, methylated; U, unmethylated; F, forward; R, reverse.

function maintenance. The present study aimed to provide novel information to improve current understanding of the development of GCT, and to potentially identify biomarkers for the early detection of GCT.

Materials and methods

Patients and samples. In the present study, 31 patients with GCTs were recruited from Shandong University Qilu Hospital (Jinan, China) and Shanxian Central Hospital (Heze, China) between 2010 and 2013. All patients were diagnosed histologically with GCT, and all tissue specimens were reconfirmed by pathologists in the Department of Pathology, Qilu Hospital, which resulted in 30 cases being available for use in the study. The present study also included tissues from 30 patients with follicular cysts, which were selected as a control. Clinicopathological data from each patient, including age, tumor size, Federation of Gynecology and Obstetrics (FIGO) stage and postoperative recurrence, were collected from medical records. The present study was approved by the Ethics Committee of Shandong University School of Medicine (Jinan, China) and the patients or their guardians provided signed informed consent prior to involvement in the investigation. Written informed consent was obtained from patients.

Methylation-specific polymerase chain reaction (MSP). A total of 31 patients with GCTs were recruited from Shandong University Qilu Hospital (Jinan, China) and Shanxian Central Hospital (Heze, China) between 2010 and 2013. All patients were diagnosed histologically with GCT, and all tissue specimens were reconfirmed by pathologists in the Department of Pathology, Qilu Hospital. One tissue wax block was not large enough, which resulted in only 30 cases being available for use in the present study. For MSP, two 8-μm tissue sections were prepared from the 30 paraffin-embedded tissue blocks and deparaffinized in xylene, following which tumor cells were dissected from sections for genomic DNA extraction. Specifically, genomic DNA was extracted using a Genomic DNA Purification kit (Qiagen, Hilden, Germany) and subjected to bisulfite conversion using a CpGenome DNA Modification kit (Intergen Co., Purchase, NY, USA), according to the manufacturer's protocols. Subsequently, 2 μl of the modified DNA (50 ng) was subjected to PCR amplification in a 50 μl volume reaction [0.25 μl Taq polymerase, 5 μl 10X PCR buffer; 4 μl dNTP mix (2.5 mM); 0.5 μl forward and reverse primers; 50 ng DNA template made up to the volume in Milli Q water] under the following conditions: 45 cycles at an annealing temperature of 58˚C for 45 sec (*CDH13*), 60˚C for 45 sec (*DKK3*) or 58˚C for 45 sec (*FOXL2*), and primer extension at 72˚C. All PCR amplifications were performed with positive controls for unmethylated and methylated alleles, and DNA-free empty controls. The PCR amplification kit was purchased from Eppendorf AG (Hamburg, Germany). The primers (Sangon Biotech., Co., Ltd., Shanghai, China) for each gene promoter methylation were designed according to previous reports (12-14) by first identifying the methylated- and unmethylated-specific sequences, respectively, and subsequent synthesis for MSP amplification of the *CDH13, DKK3* and *FOXL2* genes (Table I). The PCR products were then separated on 3% agarose gels and visualized using ethidium bromide staining under an UV-3000 ultraviolet light box.

Immunohistochemistry. Immunohistochemistry was performed to detect the protein expression of EZH2 in the tissue samples. In brief, 3-μm thick tissue sections were prepared from the paraffin-embedded tissue blocks, deparaffinized in xylene and rehydrated in a series of ethanol. The sections were then incubated with 0.5% TritonX-100 for 30 min at room temperature to ensure that the antibody entered the nuclei. The sections then underwent epitope retrieval in a steam cooker in 0.01 M citric buffer (pH 6.0) for 15 min at 100˚C. The slides were subsequently washed with phosphate-buffered saline (pH 7.4) three times for 5 min. The slides were immersed in 3% H_2O_2 methanol solution (freshly prepared) for 10 min. The slides were subsequently

Table II. Comparison of methylation rates of *CDH13*, *CKK3* and *FOXL-2* promoters between GCT and follicular cyst tissues.

Gene	GCT (n=30)	Follicular cyst (n=30)	χ^2	P-value[a]
CDH13	86.67 (26)	23.33 (7)	21.70	<0.001
DKK3	80 (24)	26.67 (8)	17.14	<0.001
FOXL2	70 (21)	20 (6)	17.38	<0.001

[a]χ^2 test compared with normal endometrium. GCT, granulosa cell tumor; CDH13, cadherin 13; DKK3, dickkopf WNT signaling pathway inhibitor 3; FOXL2, forkhead box L2.

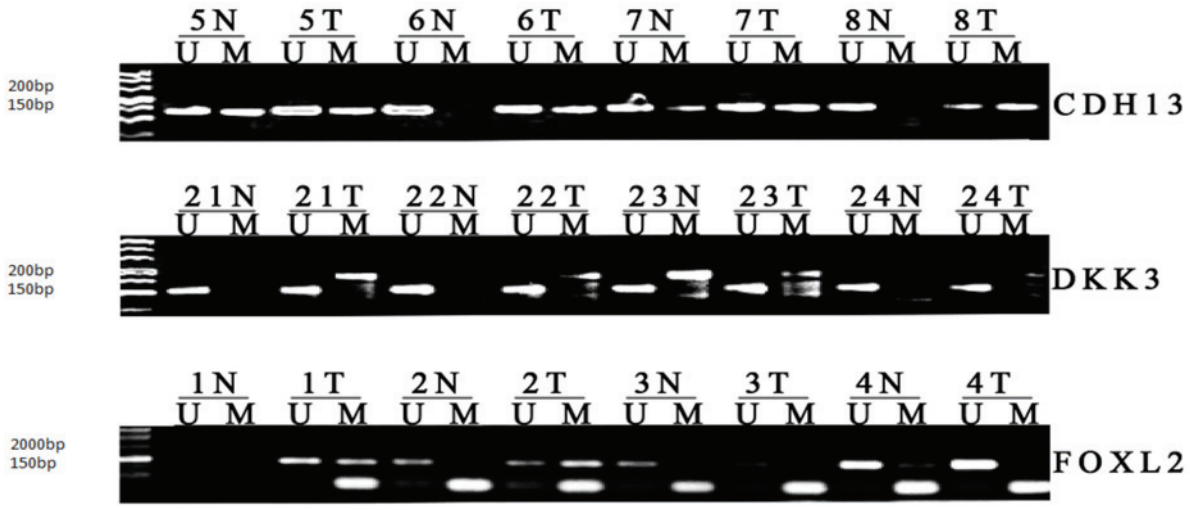

Figure 1. MSP analysis of *CDH13, DKK3* and *FOXL2* promoter methylation in GCT and follicular cyst tissues. The MSP products in the M lanes indicate the presence of methylated alleles, whereas those in the U lanes indicate the presence of unmethylated alleles. MSP, methylation-specific polymerase chain reaction; T, GCT; N, follicular cyst; CDH13, cadherin 13; DKK3, dickkopf WNT signaling pathway inhibitor 3; FOXL2, forkhead box L2; U, unmethylated; M, methylated.

washed as above. Following being blocked in normal serum for 30 min, the sections were incubated with anti-EZH2 antibody at a dilution of 1:100 (cat. no. 5246; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. The following day, the sections were washed with phosphate-buffered saline (PBS) three times and further incubated with a rabbit anti-human secondary antibody Dako (Glostrup, Denmark; cat. no. K5007) at room temperature for 30 min. The positive signal was visualized using diaminobenzidine as the chromogen. Breast cancer tissue sections (Shanxian Central Hospital; patient was diagnosed as infiltrating ductal carcinoma) were used as a positive control, and PBS was used as a negative control. The stained sections were reviewed and scored under a light microscope (BX43; Olympus, Tokyo, Japan) by two investigators for staining intensity and percentage of staining (15). The staining intensity score was recorded as follows: Absent, 0; weak, 1; moderate, 2; strong, 3. The percentage of positive cells was recorded as follows: Absent, 0; ≤10%, 1; 11-50%, 2; 51-≤80%, 3; >80 %, 4. These two scores were then multiplied to obtain a staining index. If the staining index was ≥3, the case was considered positive.

Statistical analysis. All statistical analyses were performed using SAS version 9.1 software (SAS Institute, Inc., Cary, NC, USA). The frequencies of methylation were compared using χ^2 test or Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference. All tests were two-sided.

Results

Differential methylation status of CDH13, DKK3 and FOXL2 promoters in GCTs. In the present study, the methylation status of the *CDH13*, *DKK3* and *FOXL2* promoters in the GCT tissue specimens were assessed and compared with those in follicular cyst specimens. Table II summarizes the methylation rates of these three genes in the GCTs, compared with the follicular cysts. Representative examples of the MSP data are shown in Fig. 1. Significant differences were found in the methylation of the *CDH13*, *DKK3* and *FOXL2* promoters in the GCT tissues, compared with the follicular cyst tissues (P<0.001). The associations between gene methylation and clinicopathological parameters, including age, tumor size, FIGO stage and postoperative recurrence, were also analyzed (Table III), however, no significant associations were observed.

Differential protein expression of EZH2 in GCT tissues. The immunohistochemical staining showed that EZH2 protein was localized in the nuclei of the positive tumor cells. Representative examples of the immunohistochemical

Table III. Association between *CDH13*, *DKK3* and *FOXL2* methylation and clinicopathological data from patients with granulosa cell tumors.

		DKK3 (M)		*CDH13* (M)		*FOXL2* (M)		*EZH2* (M)	
Clinical feature	n	n (%)	P-value[a]	n (%)	P-value[a]	n (%)	P-value[a]	n (%)	P-value[a]
Age (years)			1.00		0.55		1.00		1.00
<40	8	7 (87.5)		8 (100.0)		6 (75.0)		3 (37.5)	
≥40	22	17 (77.3)		18 (81.8)		15 (68.2)		8 (36.4)	
Tumor size (cm)			0.37		1.00		0.68		0.69
≥10	19	14 (73.7)		16 (84.2)		14 (73.3)		6 (31.6)	
<10	11	10 (90.9)		10 (90.9)		7 (63.6)		5 (45.5)	
FIGO stage			0.57		0.55		1.00		0.64
I	24	20 (83.3)		20 (83.3)		17 (70.8)		8 (33.3)	
II-III	6	4 (66.7)		6 (100.0)		4 (66.7)		3 (50.0)	
Recurrence			0.50		1.00		0.53		0.53
Yes	3	2 (66.7)		3 (100.0)		3 (100.0)		2 (44.4)	
No	27	22 (81.5)		23 (85.2)		18 (66.7)		9 (33.3)	

[a]Fisher's exact test. CDH13, cadherin 13; DKK3, dickkopf WNT signaling pathway inhibitor 3; FOXL2, forkhead box L2; (M), methylated; EZH2, enhancer of zeste homolog 2; FIGO, Federation of Gynecology and Obstetrics.

Table IV. Comparison of the expression of enhancer of zeste homolog 2 between GCT and follicular cyst tissues.

	EZH2				
Tissue	+ (n)	- (n)	%	χ^2	P-value[a]
GCT	11	19	36.7		
Follicular cyst	0	30	0	13.5	<0.001

[a]χ^2 test compared with normal endometrium. GCT, granulosa cell tumor; EZH2, enhancer of zeste homolog 2.

data are shown in Fig. 2. It was found that the expression of EZH2 was higher in the GCT tissues, compared with the follicular cysts (Table IV), however, no associations were found between the expression of EZH2 and the clinicopathological parameters of the patients with GCT with respect to age, tumor size, FIGO stage and metastasis (Table II). The present study then examined the associations between the expression of EZH2 and methylation of the *CDH13*, *DKK3* and *FOXL2* gene promoters, however, no positive associations were found (Table V).

Discussion

GCTs are a relatively rare type of malignancy in the ovary, and are inconsistent in size, ranging between small spots and large masses, with an average diameter of 10 cm. Although the clinical appearance, symptoms and management are similar to those of epithelial ovarian tumors, the mechanism underlying the development and progression of GCT may be different from other types of ovarian cancer (1). Thus, an improved understanding of the mechanism underlying the development and progression of GCT may lead to improved options for the early detection, prevention and treatment of GCT clinically (16). To date, clinical prognostic indicators rely predominantly on clinicopathological variables, including patient age, tumor stage and grade. Thus, the identification of molecular or epigenetic markers may provide biological insight and serve as a critical tool in the successful treatment of GCT. The present study assessed the methylation status of the *CDH13*, *DKK3* and *FOXL2* promoters, and found that the promoters of these three genes were significantly hypermethylated in the GCT tissues, compared with the follicular cyst tissues. Expression of the EZH2 protein was also high in the GCT tissues, however, no associations were found between these alterations and the clinicopathological data from the patients with GCT. Further investigations with a larger sample size are required to confirm these findings. As GCT is a relatively rare type of tumor, a consortium of different cancer centers or hospitals may be required to obtain sufficient numbers of tissue samples to facilitate further molecular investigations.

Although the cause and pathogenesis of GCTs remain to be elucidated, GCTs are similar to the majority of other types of human cancer, the development of which involves gene mutation and promoter methylation, and epigenetic modifications of genomic DNA. The accumulation of genetic abnormalities and epigenetic alterations lead to the malignant transformation of normal cells. To date, the known primary human epigenetic modifications include alterations of DNA methylation status in CpG islands and covalent modifications of histone tails. Accumulating evidence suggests that more genes are affected by aberrant epigenetic alterations than genetic mutations in human carcinogenesis (6,7,17). Thus, promoter methylation-induced silencing of tumor

Table V. Association between the expression of EZH2 and methylation of the *CDH13*, *DKK3* and *FOXL2* promoters.

	DKK3			*CDH13*			*FOXL2*		
EZH2	M	U	P-value[a]	M	U	P-value[a]	M	U	P-value[a]
Positive (n)	9	2		9	2		9	2	
Negative (n)	15	4	1.00	17	2	0.61	12	7	0.41

[a]Fisher's exact test of the association between gene methylation and the protein expression of EZH2. M, methylated; U, unmethylated; EZH2, enhancer of zeste homolog 2; *CDH13*, cadherin 13; *DKK3*, dickkopf WNT signaling pathway inhibitor 3; *FOXL2*, forkhead box L2.

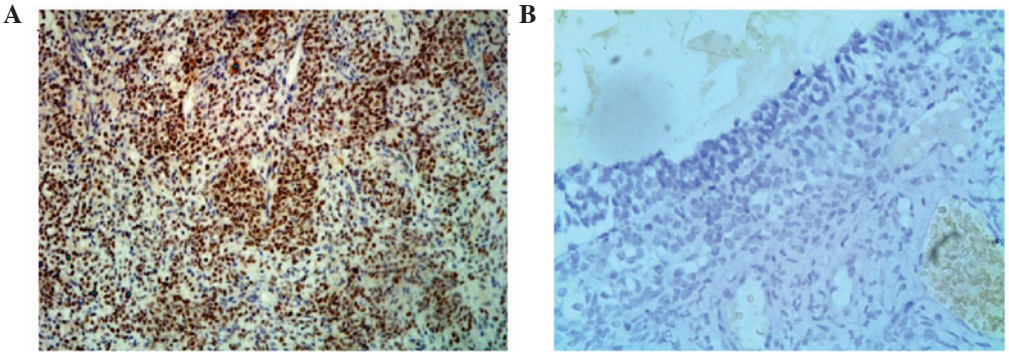

Figure 2. Immunohistochemical analysis of the protein expression of enhancer of zeste homolog 2 in (A) granulosa cell tumor (positive) and (B) follicular cyst tissues (negative) (magnification, x200).

suppressor genes has been suggested as a key mechanism in the development of several types of human cancer. Aberrant gene methylation is also one of the earliest molecular alterations occurring during tumorigenesis and may be used as a marker for early tumor detection. As methylation of the promoter region is a reversible process, the detection of gene methylation levels may provide guidance for individualized chemotherapy. The present study showed for the first time, to the best of our knowledge, methylation of the *CDH13*, *DKK3* and *FOXL2* gene promoters in GCT tissue samples, with no similar findings reported previously. Friedrichs *et al* (15) first reported a specific pattern of CpG island hypermethylation in different types of human cancer. In GCTs, the detection of different gene promoter methylation has been shown in cell lines and in a limited number of tumor tissues, with the most frequently methylated gene promoters being p16 and ER-α (40%), BRCA1 and RASSF1A (36%), MGMT (32.5%) and hMLH1 and FHIT (28%) (16,18,19).

CDH13 is a cell adherence protein of a unique cadherin superfamily member and functions to mediate intracellular signaling in vascular cells. Emerging evidence indicates that *CDH13* is a candidate tumor suppressor in several types of human cancer, including breast and lung cancer (12,20-26), colorectal cancer (21,27), hepatocellular carcinoma (28), bladder cancer (29), cervical cancer (30) and ovarian cancer (31-34). Previous studies have showed that *CDH13* promoter methylation is a frequent event in cancer, which is associated with unfavorable tumor features, increased risk of recurrence and poorer survival rates, and has been suggested as an independent predictor for tumor recurrence and progression (26,29). *DKK3* is a secreted protein, which is involved in embryonic development through its interactions with the Wnt signaling pathway. The expression of *DKK3* is reduced in a variety of cancer cell lines and may function as a tumor suppressor gene by antagonizing Wnt signaling (35-37). Epigenetic silencing of *DKK3* has been observed to disrupt normal Wnt/β-catenin signaling and apoptosis regulation (38). *DKK3* methylation has been frequently detected in a broad range of types of cancer and appears to be important in tumor development (13,37-42). *FOXL2* is a member of the forkhead transcription factor family and functions as an essential regulator of ovarian maintenance. *FOXL2* protein is expressed in the pituitary gonadotrope, thyrotrope cells and ovarian granulosa cells, and is required for commitment to ovary differentiation (43,44). *FOXL2* mutations are associated with syndromic and non-syndromic ovarian failure, and occurs in ovary GSTs with a mutation rate at *FOXL2* (402 C->G) of 97% in adult GCT (45-50). Tran *et al* showed that the CpG island of the murine *FOXL2* proximal promoter was differentially methylated in primary and immortalized cells (51). The *FOXL2* promoter was also abnormally methylated in non-small cell lung cancer (52). In the present study, the methylation statuses of three gene promoters in GCT tissues were detected, and the results demonstrated that promoter methylation was associated with the development of GCT, but not with its progression. Further investigations aim to investigate the underlying molecular mechanism for silencing the expression of these three genes in GCT.

Histone modifications are considered to regulate genetic programs in normal cells, but are altered in cancer cells. The methylation of histone H3 at lysine 27 silences gene expression, which induces transcriptional repression and is

thus involved in controlling gene expression patterns (53,54). EZH2 is a methyltransferase and a component of the polycomb repressive complex 2, which is essential in the epigenetic maintenance of the H3K27me3 repressive chromatin mark (55). The abnormal expression of EZH2 has been associated with aggressive tumor subgroups, disease-free survival rates and overall survival rates in patients with cutaneous melanoma, and in cancer of the endometrium, prostate, breast, colorectal and ovary (11,56-59). The present study was the first, to the best of our knowledge, to detect the expression of EZH2 in GCT and found 11 positive cases in 30 GCT tissue samples (36.7%), compared with follicular cyst tissue samples, in which no positive cases were found. In addition, a previous study linked EZH2 to gene silencing in association with the maintenance of DNA methylation (58). EZH2 may affect DNA methylation by direct interaction with DNA methyltransferases, however, the majority of H3K27me3-marked genes lack DNA methylation in embryonic stem cells, indicating that EZH2 recruitment may not be sufficient to promote DNA methylation (60). In the present study, no associations were found between the expression of EZH2 and methylation of the *CDH13*, *DKK3* and *FOXL2* promoters. Thus, further investigation is required to assess the functions of the EZH2 protein in GCTs.

The results of the present study were proof-of-principle, and future investigations with a larger sample size are required to verify the findings. Future investigations aim to assess how the methylation of the *CDH13*, *DKK3* and *FOXL2* gene promoters affects the expression of their proteins, and how these proteins contribute to the development of GCT. Whether the altered methylation status of these gene promoters can be detected as biomarkers for the early detection of GCT also requires investigation.

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References

1. Sun HD, Lin H, Jao MS, Wang KL, Liou WS, Hung YC, Chiang YC, Lu CH, Lai HC and Yu MH: A long-term follow-up study of 176 cases with adult-type ovarian granulosa cell tumors. Gynecol Oncol 124: 244-249, 2012.
2. Lee YK, Park NH, Kim JW, Song YS, Kang SB and Lee HP: Characteristics of recurrence in adult-type granulosa cell tumor. Int J Gynecol Cancer 18: 642-647, 2008.
3. Hashem IAT, Yaqoob I, Anuar NB, Mokhtar S, Gani A and Ullah Khan S: The rise of 'big data' on cloud computing: Review and open research issues. Inform Syst 47: 98-115, 2015.
4. Gomes A, Reis-Silva M, Alarcão A, Couceiro P, Sousa V and Carvalho L: Promoter hypermethylation of DNA repair genes MLH1 and MSH2 in adenocarcinomas and squamous cell carcinomas of the lung. Rev Port Pneumol 20: 20-30, 2014.
5. Amente S, Lania L and Majello B: Epigenetic reprogramming of Myc target genes. Am J Cancer Res 1: 413-418, 2011.
6. Nishida N, Kudo M, Nagasaka T, Ikai I and Goel A: Characteristic patterns of altered DNA methylation predict emergence of human hepatocellular carcinoma. Hepatology 56: 994-1003, 2012.
7. Ling Q, Shi W, Huang C, Zheng J, Cheng Q, Yu K, Chen S, Zhang H, Li N and Chen M: Epigenetic silencing of dual oxidase 1 by promoter hypermethylation in human hepatocellular carcinoma. Am J Cancer Res 4: 508-517, 2014.
8. Sun D, Zhang Z, Van Do N, Huang G, Ernberg I and Hu L: Aberrant methylation of CDH13 gene in nasopharyngeal carcinoma could serve as a potential diagnostic biomarker. Oral Oncol 43: 82-87, 2007.
9. Kim JS, Han J, Shim YM, Park J and Kim DH: Aberrant methylation of H-cadherin (CDH13) promoter is associated with tumor progression in primary nonsmall cell lung carcinoma. Cancer 104: 1825-1833, 2005.
10. Putku M, Kals M, Inno R, Kasela S, Org E, Kožich V, Milani L and Laan M: CDH13 promoter SNPs with pleiotropic effect on cardiometabolic parameters represent methylation QTLs. Hum Genet 134: 291-303, 2015.
11. Yoo KH and Hennighausen L: EZH2 methyltransferase and H3K27 methylation in breast cancer. Int J Biol Sci 8: 59-65, 2012.
12. Kontic M, Stojsic J, Jovanovic D, Bunjevacki V, Ognjanovic S, Kuriger J, Puumala S and Nelson HH: Aberrant promoter methylation of CDH13 and MGMT genes is associated with clinicopathologic characteristics of primary non-small-cell lung carcinoma. Clin Lung Cancer 13: 297-303, 2012.
13. Kloten V, Becker B, Winner K, Schrauder MG, Fasching PA, Anzeneder T, Veeck J, Hartmann A, Knüchel R and Dahl E: Promoter hypermethylation of the tumor-suppressor genes ITIH5, DKK3, and RASSF1A as novel biomarkers for blood-based breast cancer screening. Breast Cancer Res 15: R4, 2013.
14. Zhou XY, Sun JF, He YH, Zhang HY, Yu J, Guo SC, Cai Y, Hu XC and Zhu JD: Correlation of the methylation status of CpG islands in the promoter region of 10 genes with the 5-Fu chemosensitivity in 3 breast cancer cell lines. Zhonghua Zhong Liu Za Zhi 32: 328-333, 2010.
15. Friedrichs K, Gluba S, Eidtmann H and Jonat W: Overexpression of p53 and prognosis in breast cancer. Cancer 72: 3641-3647, 1993.
16. Dhillon VS, Young AR, Husain SA and Aslam M: Promoter hypermethylation of MGMT, CDH1, RAR-beta and SYK tumour suppressor genes in granulosa cell tumours (GCTs) of ovarian origin. Br J Cancer 90: 874-881, 2004.
17. Chen H, Zhang C, Sheng Y, Yao S, Liu Z, Zhang C and Zhang T: Frequent SOCS3 and 3OST2 promoter methylation and their epigenetic regulation in endometrial carcinoma. Am J Cancer Res 5: 180-190, 2014.
18. Dhillon VS, Aslam M and Husain SA: The contribution of genetic and epigenetic changes in granulosa cell tumors of ovarian origin. Clin Cancer Res 10: 5537-5545, 2004.
19. Dhillon VS, Shahid M and Husain SA: CpG methylation of the FHIT, FANCF, cyclin-D2, BRCA2 and RUNX3 genes in Granulosa cell tumors (GCTs) of ovarian origin. Mol Cancer 3: 33, 2004.
20. Toyooka KO, Toyooka S, Virmani AK, Sathyanarayana UG, Euhus DM, Gilcrease M, Minna JD and Gazdar AF: Loss of expression and aberrant methylation of the CDH13 (H-cadherin) gene in breast and lung carcinomas. Cancer Res 61: 4556-4560, 2001.
21. Toyooka S, Toyooka KO, Harada K, Miyajima K, Makarla P, Sathyanarayana UG, Yin J, Sato F, Shivapurkar N, Meltzer SJ and Gazdar AF: Aberrant methylation of the CDH13 (H-cadherin) promoter region in colorectal cancers and adenomas. Cancer Res 62: 3382-3386, 2002.
22. Xu J, Shetty PB, Feng W, Chenault C, Bast RC Jr, Issa JP, Hilsenbeck SG and Yu Y: Methylation of HIN-1, RASSF1A, RIL and CDH13 in breast cancer is associated with clinical characteristics, but only RASSF1A methylation is associated with outcome. BMC Cancer 12: 243, 2012.
23. Zhai X and Li SJ: Methylation of RASSF1A and CDH13 genes in individualized chemotherapy for patients with non-small cell lung cancer. Asian Pac J Cancer Prev 15: 4925-4928, 2014.
24. Zhai X and Li SJ: Methylation of RASSF1A and CDH13 genes in individualized chemotherapy for patients with non-small cell lung cancer. Asian Pac J Cancer Prev 15: 4925-4928, 2014.
25. Zhong YH, Peng H, Cheng HZ and Wang P: Quantitative assessment of the diagnostic role of CDH13 promoter methylation in lung cancer. Asian Pac J Cancer Prev 16: 1139-1143, 2015.
26. Xue R, Yang C, Zhao F and Li D: Prognostic significance of CDH13 hypermethylation and mRNA in NSCLC. Onco Targets Ther 7: 1987-1996, 2014.
27. Hibi K, Kodera Y, Ito K, Akiyama S and Nakao A: Aberrant methylation of HLTF, SOCS-1 and CDH13 genes is shown in colorectal cancers without lymph node metastasis. Dis Colon Rectum 48: 1282-1286, 2005.
28. Riou P, Saffroy R, Chenailler C, Franc B, Gentile C, Rubinstein E, Resink T, Debuire B, Piatier-Tonneau D and Lemoine A: Expression of T-cadherin in tumor cells influences invasive potential of human hepatocellular carcinoma. FASEB J 20: 2291-2301, 2006.

29. Lin YL, Xie PG and Ma JG: Aberrant methylation of CDH13 is a potential biomarker for predicting the recurrence and progression of non muscle invasive bladder cancer. Med Sci Monit 20: 1572-1577, 2014.
30. Abudukadeer A, Bakry R, Goebel G, Mutz-Dehbalaie I, Widschwendter A, Bonn GK and Fiegl H: Clinical relevance of CDH1 and CDH13 DNA-methylation in serum of cervical cancer patients. Int J Mol Sci 13: 8353-8363, 2012.
31. Wu Q, Lothe RA, Ahlquist T, Silins I, Tropé CG, Micci F, Nesland JM, Suo Z and Lind GE: DNA methylation profiling of ovarian carcinomas and their in vitro models identifies HOXA9, HOXB5, SCGB3A1, and CRABP1 as novel targets. Mol Cancer 6: 45, 2007.
32. Makarla PB, Saboorian MH, Ashfaq R, Toyooka KO, Toyooka S, Minna JD, Gazdar AF and Schorge JO: Promoter hypermethylation profile of ovarian epithelial neoplasms. Clin Cancer Res 11: 5365-5369, 2005.
33. Rathi A, Virmani AK, Schorge JO, Elias KJ, Maruyama R, Minna JD, Mok SC, Girard L, Fishman DA and Gazdar AF: Methylation profiles of sporadic ovarian tumors and nonmalignant ovaries from high-risk women. Clin Cancer Res 8: 3324-3331, 2002.
34. Kawakami M, Staub J, Cliby W, Hartmann L, Smith DI and Shridhar V: Involvement of H-cadherin (CDH13) on 16q in the region of frequent deletion in ovarian cancer. Int J Oncol 15: 715-720, 1999.
35. Yu J, Tao Q, Cheng YY, Lee KY, Ng SS, Cheung KF, Tian L, Rha SY, Neumann U, Röcken C, *et al*: Promoter methylation of the Wnt/beta-catenin signaling antagonist Dkk-3 is associated with poor survival in gastric cancer. Cancer 115: 49-60, 2009.
36. Ying J, Li H, Yu J, Ng KM, Poon FF, Wong SC, Chan AT, Sung JJ and Tao Q: WNT5A exhibits tumor-suppressive activity through antagonizing the Wnt/beta-catenin signaling, and is frequently methylated in colorectal cancer. Clin Cancer Res 14: 55-61, 2008.
37. Hayashi T, Asano H, Toyooka S, Tsukuda K, Soh J, Shien T, Taira N, Maki Y, Tanaka N, Doihara H, *et al*: DNA methylation status of REIC/Dkk-3 gene in human malignancies. J Cancer Res Clin Oncol 138: 799-809, 2012.
38. Voorham QJ, Janssen J, Tijssen M, Snellenberg S, Mongera S, van Grieken NC, Grabsch H, Kliment M, Rembacken BJ, Mulder CJ, *et al*: Promoter methylation of Wnt-antagonists in polypoid and nonpolypoid colorectal adenomas. BMC Cancer 13: 603, 2013.
39. Yin DT, Wu W, Li M, Wang QE, Li H, Wang Y, Tang Y and Xing M: DKK3 is a potential tumor suppressor gene in papillary thyroid carcinoma. Endocr Relat Cancer 20: 507-514, 2013.
40. Kang WS, Cho SB, Park JS, Lee MY, Myung SC, Kim WY, Lee SH, Kim DH and Lee EJ: Clinico-epigenetic combination including quantitative methylation value of DKK3 augments survival prediction of the patient with cervical cancer. J Cancer Res Clin Oncol 139: 97-106, 2013.
41. Liang L, He H, Lv R, Zhang M, Huang H, An Z and Li S: Preliminary mechanism on the methylation modification of Dkk-1 and Dkk-3 in hepatocellular carcinoma. Tumour Biol 36: 1245-1250, 2015.
42. Tao L, Huang G, Chen Y and Chen L: DNA methylation of DKK3 modulates docetaxel chemoresistance in human nonsmall cell lung cancer cell. Cancer Biother Radiopharm 30: 100-106, 2015.
43. Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G and Schlessinger D: Foxl2 is required for commitment to ovary differentiation. Hum Mol Genet 14: 2053-2062, 2005.
44. Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC and Treier M: The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development 131: 933-942, 2004.
45. Rosario R, Araki H, Print CG and Shelling AN: The transcriptional targets of mutant FOXL2 in granulosa cell tumours. PLoS One 7: e46270, 2012.
46. Shah SP, Köbel M, Senz J, Morin RD, Clarke BA, Wiegand KC, Leung G, Zayed A, Mehl E, Kalloger SE, *et al*: Mutation of FOXL2 in granulosa-cell tumors of the ovary. N Engl J Med 360: 2719-2729, 2009.
47. Jamieson S, Butzow R, Andersson N, Alexiadis M, Unkila-Kallio L, Heikinheimo M, Fuller PJ and Anttonen M: The FOXL2 C134W mutation is characteristic of adult granulosa cell tumors of the ovary. Mod Pathol 23: 1477-1485, 2010.
48. Kim MS, Hur SY, Yoo NJ and Lee SH: Mutational analysis of FOXL2 codon 134 in granulosa cell tumour of ovary and other human cancers. J Pathol 221: 147-152, 2010.
49. D'Angelo E, Mozos A, Nakayama D, Espinosa I, Catasus L, Muñoz J and Prat J: Prognostic significance of FOXL2 mutation and mRNA expression in adult and juvenile granulosa cell tumors of the ovary. Mod Pathol 24: 1360-1367, 2011.
50. Oseto K, Suzumori N, Nishikawa R, Nishikawa H, Arakawa A, Ozaki Y, Asai H, Kawai M, Mizuno K, Takahashi S, *et al*: Mutational analysis of FOXL2 p.C134W and expression of bone morphogenetic protein 2 in Japanese patients with granulosa cell tumor of ovary. J Obstet Gynaecol Res 40: 1197-1204, 2014.
51. Tran S, Wang Y, Lamba P, Zhou X, Boehm U and Bernard DJ: The CpG island in the murine foxl2 proximal promoter is differentially methylated in primary and immortalized cells. PLoS One 8: e76642, 2013.
52. Zhao Y, Zhou H, Ma K, Sun J, Feng X, Geng J, Gu J, Wang W, Zhang H, He Y, *et al*: Abnormal methylation of seven genes and their associations with clinical characteristics in early stage non-small cell lung cancer. Oncol Lett 5: 1211-1218, 2013.
53. Sharma S, Kelly TK and Jones PA: Epigenetics in cancer. Carcinogenesis 31: 27-36, 2010.
54. Viré E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, *et al*: The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439: 871-874, 2006.
55. Cao R and Zhang Y: The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 14: 155-164, 2004.
56. Hoffmann MJ, Engers R, Florl AR, Otte AP, Muller M and Schulz WA: Expression changes in EZH2, but not in BMI-1, SIRT1, DNMT1 or DNMT3B are associated with DNA methylation changes in prostate cancer. Cancer Biol Ther 6: 1403-1412, 2007.
57. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, Salvesen HB, Otte AP and Akslen LA: EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol 24: 268-273, 2006.
58. Wang J, Yu L, Cai J, Jia J, Gao Y, Liang M and Wang Z: The role of EZH2 and DNA methylation in hMLH1 silencing in epithelial ovarian cancer. Biochem Biophys Res Commun 433: 470-476, 2013.
59. Kodach LL, Jacobs RJ, Heijmans J, van Noesel CJ, Langers AM, Verspaget HW, Hommes DW, Offerhaus GJ, van den Brink GR and Hardwick JC: The role of EZH2 and DNA methylation in the silencing of the tumour suppressor RUNX3 in colorectal cancer. Carcinogenesis 31: 1567-1575, 2010.
60. Rush M, Appanah R, Lee S, Lam LL, Goyal P and Lorincz MC: Targeting of EZH2 to a defined genomic site is sufficient for recruitment of Dnmt3a but not de novo DNA methylation. Epigenetics 4: 404-414, 2009.