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Hypermethylation and aberrant expression of Wnt-antagonist family genes in gastric cardia adenocarcinoma

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The canonical Wnt signalling pathway plays a key role during embryogenesis and pathogenesis of various types of tumors. Recently, several studies have shown that the promoter hypermethylation of Wnt-antagonist genes, including *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3*, have been certified to contribute to the tumorigenesis of several cancers.

The aim of this study was to investigate the promoter methylation of Wnt-antagonist genes in gastric cardia adenocarcinoma (GCA) and corresponding adjacent non-cancerous tissues, and to establish the possible relationship between DNA methylation status and the pathogenesis of GCA.

MSP, RT-PCR methods were applied respectively to examine the CpG methylation of the Wnt-antagonist genes and its mRNA expression in tumors and corresponding non-cancerous tissues, and immunohistochemistry method was used to determine protein expression of β-catenin(the key factor of the Wnt signalling pathway) and cyclin D1(the target gene of this pathway).

The frequency of promoter methylation of *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3* genes in GCA tumor tissues were 78.7%(74/94), 76.6%(72/94), 70.2%(66/94), 77.1%(73/94), 61.7%(58/94) and 21.3%(20/94), respectively, which were significantly higher than those in adjacent non-cancerous tissues. Furthermore, the frequencies of silenced mRNA expression of these six genes in GCA tumor tissues were significantly higher than those in adjacent non-cancerous tissues. Methylation levels of these six genes were all correlated with loss of mRNA expression. The ectopic expression of β-catenin and cyclin D1 was significantly more frequent in GCA tumor tissues than that in adjacent non-cancerous tissues and correlated with each Wnt-antagonist genes hypermethylation status.

Epigenetic silencing of Wnt-antagonist genes expression by promoter hypermethylation may play an important role in gastric cardia adenocarcinoma.

Key words: Wnt-antagonist genes, DNA methylation, gastric cardiac adenocarcinoma

Gastric cardia adenocarcinoma (GCA) is one type of digestive tract cancers, which was formerly registered as esophageal cancer or gastric cancer. It has been diagnosed independently in very recent years, due to the improvement in early endoscopic screening and pathologic diagnosis [1]. China is a country with high incidence regions of GCA, especially in Taihang mountain of North China. The epidemiological data has suggested that the incidence of GCA has been increasing in recent years [2]. Exogenous factors including nutrition deficiency, unhealthy living habits, pathogenic infections, and consumption of alcohol and tobacco are generally considered as the risk factors for developing GCA in China [3, 4], however, only a subset of individuals exposed to the above listed exogenous risk factors would develop GCA, suggesting that multiple genetic and epigenetic events may contribute to the occurrence and progression of GCA. But the precise molecular mechanisms of the development and progression of GCA still remain unknown. Cancer is fundamentally a genetic and epigenetic disease that requires the accumulation of genomic alterations which inactivate tumor suppressors and activate proto-oncogenes. In addition to genetic mutation or allelic loss, epigenetic alterations including DNA methylation is now recognized as an alternative mechanism to silence tumor suppressor genes [5]. It may result in the silencing of transcription and, hence, inactivation of that gene, which may then confer growth advantages to these cells that favor cancer development.

The activation of canonical Wnt signalling pathway plays a key role during the pathogenesis of various types of tumours [6]. Acts as the key mediator of this signaling pathway, the level of ß-catenin kept low through phosphorylation and degradation in a complex with axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3ß(GSK-3ß) in normal conditions.

However, when the Wnt signaling is activated, the Wnt signaling protein binded to two receptor molecules, Frizzled proteins and lipoprotein receptor-related proteins 5 and 6 (LRP-5/6) , activates disheveled phosphoprotein, which in turn inactivates GSK-3ß. The inhibited GSK-3ß fails to phosphorylate ß-catenin, which results in uncoupling of ß-catenin from the degradation pathway [7, 8]. Consequently, ß-catenin accumulates in the cytoplasm and enters the nucleus, where it interacts with TCF/LEF family of transcription factors to control transcription and activates the target genes expression, including cyclin D1 which are involved in initiation and progression of tumor [9, 10].

In recent investigations, several antagonists of Wnt pathway have been identified and can be divided into two functional classes, the secreted frizzled-related protein (sFRP) class and the *Dickkopf* (Dkk) class [11]. As Wnt-antagonist genes, *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3* inhibited Wnt signaling by directly binding to Wnt molecules or by binding to the LRP5/LRP6 component of the Wnt receptor complex. Thus, the functional loss of Wnt antagonists can contribute to activation of the Wnt pathway and induced ectopic expression of ß-catenin. Up to now, downregulation of Wnt-antagonist genes have been identified in a variety of malignancies, including bladder [8, 11, 12], lung [13, 14], breast [15], and chronic lymphocytic leukemia [16], and even the gastric carcinoma [17, 18]and esophageal carcinoma [19]. Inactivation caused by promoter hypermethylation seems to be one of the mechanisms underlying down-regulation of the Wnt antagonists, which strongly suggests Wnt-antagonist genes function as tumor suppressor genes may play important role in tumorigenesis. However, report about promoter CpG methylation of the Wnt-antagonist genes in GCA had not been found.

Based on previous studies, we hypothesized that inactivation caused by promoter hypermethylation of the Wnt-antagonist genes may be one of the mechanism to develop GCA, so we analyzed the promoter hypermethylation status and mRNA expression of Wnt-antagonist genes in GCA patients of north China to determine the possible relationship between the methylation status of Wnt-antagonist genes and ectopic expression of ß-catenin and cyclin D1.

Methods

Patients and specimens. 94 samples of GCA were all obtained from the inpatients for surgical treatment in the Fourth Affiliated Hospital, Hebei Medical University between 2004 and 2006. 78 male patients and 16 female ones were included, mean age 53.8 years (ranged from 37 to 79 years). GCA tissues and corresponding adjacent tissues were all obtained from operations. These tissues were divided into two parallel parts, one part were frozen and stored at -80°C to extracted RNA, the other part were formalin-fixed and paraffin-embedded. Histological tumor typing was carried out on the basis of resected specimens in the Department of Pathology of the same hospital. Sample sections were stained in H&E and were examined by two experienced pathologists. All tumor tissues were adenocarcinoma with their epicenters at the gastroesophageal junction, i.e. from 1cm above until 2cm below the junction between the end of the tubular esophagus and the beginning of the saccular stomach [20]. Information on tumor-node-metastasis (TNM) classification was available from hospital recordings and pathological diagnosis. 38 of them were stage I and II (40.4%) 56 were stage III and IV (59.6%). According to the pathological phases, the cases were classified into 3 groups, 19(20.2%)of the cases were well differentiation, 26(27.7%) of them were moderate differentiation and 49(52.1%) of them were poor differentiation. The study was approved by the Ethics Committee of Hebei Cancer Institute and informed consent was obtained from all recruited subjects.

Methylation-specific PCR Genomic DNA from gastric cardia adenocarcinomas and adjacent non-cancerous sections were isolated by manual microdissected method from paraffin-embedded tissue slides using a simplified proteinase K digestion method. To examine the DNA methylation patterns, we treated genomic DNA with sodium bisulfite, as described previously [21]. Generally, CpG sites within a promoter are unmethylated, but are occasionally methylated in various cancers. Unmethylated cytosines are converted to uracil by bisulfite treatment, whereas methylated cytosine cannot be converted and remain as cytosine. Based on this potential difference in the DNA sequence between methylated and unmethylated alleles after bisulfite treatment, we designed primer sequences that could distinguish methylated from unmethylated alleles. The targeted genes used in this study were *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1,* and *Dkk-3*, which primer sequences and PCR conditions (product size and annealing temperatures) are shown in Table 1. The methylation status of the *sFRP-3* gene was not examined because it does not have any CpG islands [11]. PCR products were analyzed on 2% agarose gels with ethidium bromide and visualized under UV illumination. The positive control was used methylated genomic DNA, which treated by CpG methyltransferase (Sss I) following the manufacturer's directions (New England BioLabs, Inc, Beverly, MA). Water blank was used as a negative control. 10% of samples were repeated methylation analysis for quality control.

RT-PCR analysis. The cDNA was prepared using total RNA and stored at –80°C until used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control. The primer sequences and annealing temperatures are shown in(Tab. 1). The PCR products were separated in 2% agarose gel in electrophoresis, and quantified using an image analysis system (UVP Bioimaing systems, USA).

Immunostaining. Immunostaining of ß-catenin and cyclin D1 was performed on parallel histopathological sections from paraffin-embedded tumor section and corresponding adjacent tissues. Tissue sections were deparaffinized in xylene, rehydrated in graded alcohol. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes, followed by microwave antigen retrieval for nine minutes at 95°C in 10mM sodium citrate buffer and incubated in 1% normal goat serum to minimize non-specific protein binding. The slides were sequentially incubated with

Table1. Primer sequences and PCR conditions of Wnt-antagonist genes

Gene	Sense primer (5' → 3')	Antisense primer (5' → 3')	Product size (bp)	Annealing temperatures,
sFRP1				
M	TGTAGTTTTCGGAGTTAGTGTCGCGC	CCTACGATCGAAAACGACGCGAACG	126	65.5°C
U	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT	CTCAACCTACAATCAAAAACAACACAAACA	135	63.1°C
RT-PCR	CGAGTTTGCACTGAGGATGA	CAGCACAAGCTTCTTCAGGTC	130	54.0°C
sFRP2				
M	GGGTCGGAGTTTTTCGGAGTTGCGC	CCGCTCTCTTCGCTAAATACGACTCG	138	63.1°C
U	TTTTGGGTTGGAGTTTTTTGGAGTTGTG	AACCCACTCTCTTCACTAAATACAACT	145	55.7°C
RT-PCR	CGAGGAAGCTCCAAAGGTAT	CTCCTTCACTTTTATTTTCAGTGCAA	112	55.2°C
sFRP4				
M	GGGTGATGTTATCGTTTTTGTATCGAC	CCTCCCCTAACGTAAACTCGAAACG	111	59.1°C
U	GGGGGTGATGTTATTGTTTTTGTATTGAT	CACCTCCCCTAACATAAACTCAAAACA	115	59.2°C
RT-PCR	TCTTGCCAGTGTCCACACATC	CCTCTCTTCCCACTGTATGGA	147	55.2°C
sFRP5				
M	AAGATTTGGCGTTGGGCGGGACGTTC	ACTCCAACCCGAACCTCGCCGTACG	136	61.8°C
U	GTAAGATTTGGTGTTGGGTGGGATGTTT	CAAAACTCCAACCCAAACCTCACCATACA	141	52.7°C
RT-PCR	CGCCTCCAGTGACCAAGAT	GATGCGCATTTTGACCACAAAG	104	55.2°C
Wif-1				
M	GTTTTCGGAGTTAGTGTCGCGC	ACGATCGAAAACGACGCGAACG	119	57°C
U	GTAGTTTTTGGAGTTAGTGTTGTGT	ACCTACAATCAAAAACAACACAAACA	126	55°C
RT-PCR	TCTGTTCAAAGCCTGTCTGC	CCTTTTATTGCAGTGTCTTCCA	111	56.6°C
Dkk3				
M	GGGGCGGGCGGCGGGGC	ACATCTCCGCTCTACGCCCG	120	58°C
U	TTAGGGGTGGGTGGTGGGGT	CTACATCTCCACTCTACACCCA	126	56°C
RT-PCR	ACAGCCACAGCCTGGTGTA	CCTCCATGAAGCTGCCAAC	120	57.8°C
GAPDH	GGGAAACTGTGGCGTGAT	GTGGTCGTTGATTTCAAT	342	60.5°C

primary monoclonal ß-catenin (clone 680-781(H), Santa cruz, dilution 1:100), and CyclinD1 (clone SP4, Santa cruz, dilution 1:100) antibodies at 4°C overnight, biotinylated secondary antibody for 30 min at 37°C, 3,3'-Diaminobenzidine (Sigma, St Louis, MO) was used as the chromogen, and counterstaining was done using hematoxylin. In negative controls, the primary antibody was replaced with nonimmune serum. Immunohistochemical staining of ß-catenin showed a strong cytoplasmic/nucleus or membranous staining (Figure 1). We defined cytoplasmic/nucleus staining as ectopic expression and membranous staining as normal expression, and nucleus staining of cyclin D1 as positive expression. Protein expression in cytoplasmic/nucleus (ß-catenin) and the nuclei (cyclin D1) was evaluated according to the proportion of positive cytoplasm or nuclei to all epithelial or cancer cells (positive means >20% positive cytoplasm/nuclei, negative means 0-20% positive cytoplasm/nuclei).

Statistical analysis. All data were analyzed by the SPSS11.5 software (SPSS Company, Chicago, Illinois, USA). Statistical analysis was done using the Chi-square test. Two-sided tests were used to determine significance, and P values less than 0.05 was regarded as statistically significant for all statistic tests.

Results

Methylation analysis of Wnt-antagonist genes in GCA. The methylation analysis was successfully performed in all tumor and corresponding adjacent non-cancerous tissues. There were three status of methylation analysis result – complete methylation, only methylated DNA was amplified; incomplete methylation, both methylated and unmethylated DNA were amplified; and unmethylation, only unmethylated DNA was amplified. In addition, no methylated bands were found in negative control samples. Figure 2 illustrates representative results of methylation analysis of six Wnt-antagonist genes. Methylated bands of Wnt-antagonist genes were present in the majority of GCA samples, and less evident band was found in the adjacent non-cancerous samples. Unmethylated bands were present in most GCA and adjacent samples. As shown in Table 2 , the frequency of both complete and incomplete methylation of *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3* genes in GCA tumor tissues were 78.7%(74/94), 76.6%(72/94), 70.2%(66/94), 77.1%(73/94), 61.7%(58/94) and 21.3%(20/94), respectively, which were significantly higher than those in adjacent non-cancerous tissues($P<0.01$).

Association analysis of Wnt-antagonist genes methylation status with clinicopathologic features. As shown in Table 3**,** there was no relationship between each methylation levels of Wnt-antagonist genes and a single variable with histologic grade and TNM stage. When the six genes were combined to analysis, we found that the frequency of promoter simultaneous methylation of more than three genes was significantly higher in stage III and IV patients than those in stage I and

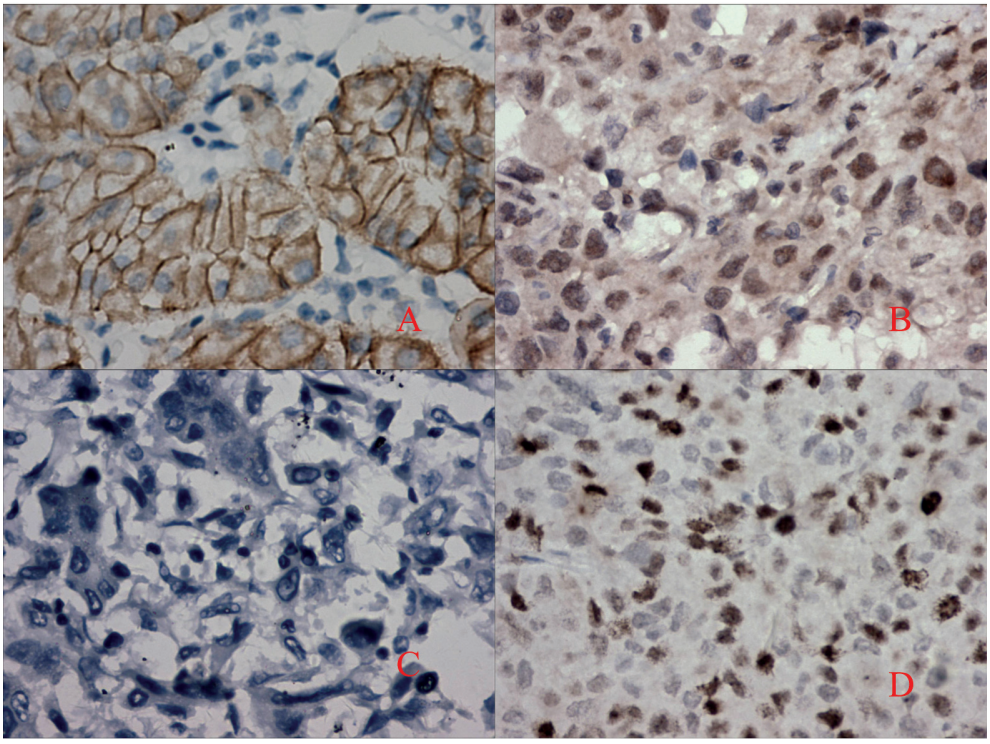

Figure 1. The expression of β-catenin and Cyclin D1 in GCA tissues (DAB ×400). A. The normal expression of β-catenin in GCA tissues; B. The ectopic expression of β-catenin in GCA tissues; C. The negative expression of Cyclin D1 in GCA tissues; D. The positive expression of Cyclin D1 in GCA tissues

II patients($p<0.05$), however, the connection of simultaneous methylation of these genes with the other investigated parameters was not found (Table 4).

mRNA expression of Wnt-antagonist genes in GCA. mRNA expression of Wnt-antagonist genes was examined by RT-PCR in 94 GCA samples and corresponding adjacent non-cancerous tissues (Figure 3). Those results were shown in Table 2. The frequencies of silenced mRNA expression of *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3* genes in GCA tumor tissues were 51.1%(48/94), 46.8%(44/94), 40.4%(38/94), 53.2%(50/94), 35.1%(33/94) and 23.6%(22/94), respectively, which were significantly higher than those in adjacent tissues. The loss of mRNA transcript expression in all investigated Wnt-antagonist genes were correlated with the relative methylation frequency in these gene promoters, as shown in Table 5. Furthermore, the inhibited frequencies of mRNA expression of Wnt-antagonist genes in complete methylation groups were significantly higher than those in incomplete methylation groups (Table 6).

Protein expression of ß-catenin, cyclin D1 in GCA. The ectopic expression of ß-catenin in GCA was 86.2% (81/94), while only 30.9%(29/94) corresponding adjacent tissues showed ectopic expression($P=0.000$). The ectopic expression of ß-catenin in Wnt-antagonist genes methylation groups was significantly higher than unmethylation groups in GCA as shown in **table 5**. The difference had

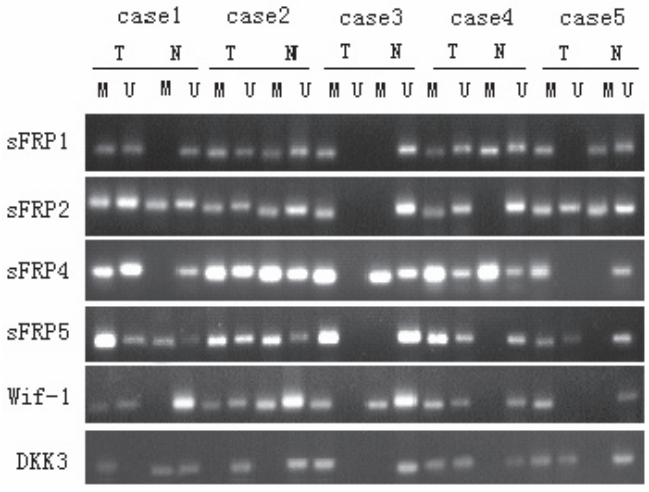

Figure 2. Methylation analysis of Wnt-antagonist genes
MSP results of the Wnt-antagonist genes in 5 matched pairs (case1-case5) of tumor tissue (T) and non-cancerous tissue (N). M: Methylated genes; U: Unmethylated genes.
the tumor is fully methylated and the non-cancerous tissue is incomplete methylated: case5 of sFRP1, case3 of sFRP4, case3 of Wif-1 and case1 of Dkk3; both tumor and non-cancerous tissue are incomplete methylated: case2, 4 of sFRP1, case1, 2, 5 of sFRP2, case2,4 of sFRP4, case1,2 of sFRP5 and case2 of Wif-1; both tumor and non-cancerous tissue are unmethylated: case1 of Wif-1 and case2 of Dkk3; tumor-specific methylation: other cases

Table 2. Methylation status and mRNA expression of Wnt-antagonist genes

gene	Methylation frequency		*p*	mRNA negative expression		*p*
	GCA	non-cancerous tissues		GCA	non-cancerous tissues	
sFRP1	78.7%(74/94)	14.9%(14/94)	0.000	51.1%(48/94)	5.3%(5/94)	0.000
sFRP 2	76.6%(72/94)	54.3%(51/94)	0.001	46.8%(44/94)	26.6%(25/94)	0.004
sFRP 4	70.2%(66/94)	9.6%(9/94)	0.000	40.4%(38/94)	4.3%(4/94)	0.000
sFRP 5	77.1%(73/94)	12.8%(12/94)	0.000	53.2%(50/94)	12.8%(12/94)	0.000
Wif-1	61.7% (58/94)	34.0% (32/94)	0.000	35.1%(33/94)	13.8%(13/94)	0.001
Dkk3	21.3% (20/94)	0% (0/94)	0.000	23.6%(22/94)	2.1%(2/94)	0.000

Table 3. The relationship between Wnt-antagonist genes methylation status and clinicopathologic features

Group	n	Sfrp1		*P*	Sfrp2		*P*	Sfrp4		*P*	Sfrp5		*P*	Wif-1		*P*	Dkk3		*P*
		M	U		M	U		M	U		M	U		M	U		M	U	
Histological grade																			
well	19	15	4		14	5		12	7		15	4		13	6		6	13	
moderate	26	18	8		19	7		16	10		18	8		14	12		7	19	
poor	49	41	8	0.360[a]	39	10	0.773[a]	38	11	0.266[a]	40	9	0.479[a]	31	18	0.579[a]	7	42	0.209[a]
TNM stage																			
I	9	5	4		4	5		3	6		6	3		2	7		1	8	
II	29	24	5		22	7		20	9		27	2		18	11		8	21	
III	41	37	4		33	8		30	11		26	15		29	12		9	32	
IV	15	8	7	0.671[b]	13	2	0.132[b]	13	2	0.091[b]	14	1	0.078[b]	9	6	0.136[b]	2	13	0.638[b]

[a] : P value among three differentiation groups

[b] : P value of stage III and IV patients against stage I and II patients

M: both completely and incompletely methylated; U: unmethylated

Table 4. Association analysis of Wnt-antagonist genes methylation status with clinicopathologic features

Group	n	Simultaneous methylation of one or two genes		*p*	Simultaneous methylation of three or four genes		*p*	Simultaneous methylation of five or six genes		*p*
		M	U		M	U		M	U	
Histological grade										
well	19	9	10		7	12		0	19	
moderate	26	5	21		9	17		3	23	
poor	49	21	28	0.239[a]	25	24	0.131[a]	10	39	0.054[a]
TNM stage										
I	9	3	6		1	8		0	9	
II	29	9	20		10	19		1	28	
III	41	14	27		19	22		5	36	
IV	15	9	6	0.350[b]	11	4	0.018[b]	7	8	0.010[b]

[a]: P value among three differentiation groups

[b]: P value of stage III and IV patients against stage I and II patients

M: both completely and incompletely methylated; U: unmethylated

reached statistical significance. Ectopic expression of ß-catenin was respectively correlated with each Wnt-antagonist genes methylation status in GCA (P<0.01). The positive expression of CyclinD1 in GCA was 73/94(77.7%), which was significantly higher than that in corresponding adjacent tissues (38/94, 40.4%) (P<0.01). The frequency of cyclinD1 protein expression in *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3* genes methylation groups were 83.8%(62/74), 84.7%(61/72), 83.3%(55/66), 94.5%(69/73), 89.7%(52/58) and 100.0%(20/20), respectively, which were significantly higher than those in unmethylation groups. Cyclin D1 hyper-expression was respectively correlated with each Wnt-antagonist gene methylation status in GCA (P<0.01) (Fig. 4).

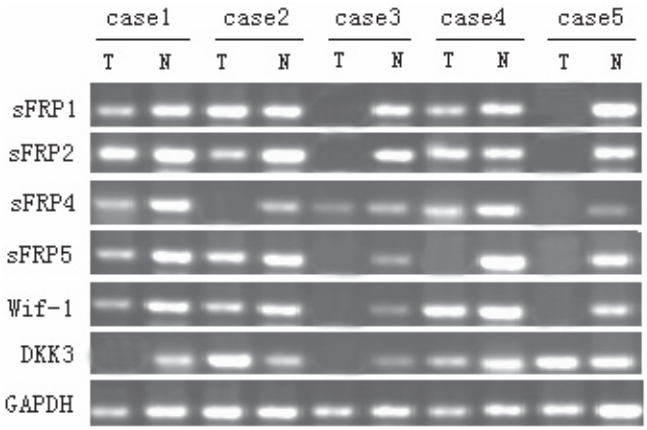

Figure 3. The mRNA expression of Wnt-antagonist genes
RT-PCR results of the Wnt-antagonist genes in 5 matched pairs (case1-case5) of tumor tissues (T) and non-cancerous tissues (N). GAPDH is shown as a control. Most of the Wnt-antagonist genes mRNA expression was lower in GCA tissues than those in non-cancerous tissues.

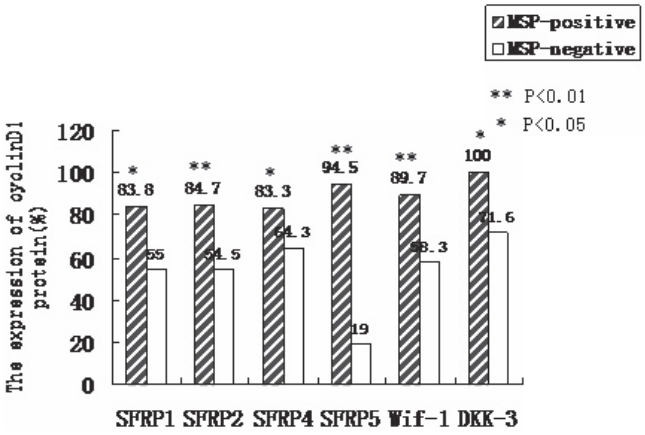

Figure.4 The protein expression of cyclinD1 in hypermethylation and unmethylation groups
Each of Wnt-antagonist genes in hypermethylation group shown the higher expression of cyclinD1 compared to unmethylation group in GCA tissues.

Table 5. Wnt-antagonist genes mRNA negative expression and β-catenin protein ectopic expression between methylation and unmethylation groups in GCA

gene	mRNA negative expression frequency		P	ectopic expression of ß-catenin		P
	methylation	unmethylation		methylation	unmethylation	
sFRP1	62.2%(46/74)	10.0%(2/20)	0.000	100.0%(74/74)	35.0%(7/20)	0.000
sFRP 2	52.8%(38/72)	27.3%(6/22)	0.036	91.7%(66/72)	68.2%(15/22)	0.015
sFRP 4	47.0%(31/66)	25.0%(7/28)	0.047	98.5%(65/66)	57.1%(16/28)	0.000
sFRP 5	63.0%(46/73)	19.0%(4/21)	0.000	95.9%(70/73)	52.4%(11/21)	0.000
Wif-1	46.6%(27/58)	16.7%(6/36)	0.003	93.1%(54/58)	75.0 %(27/36)	0.013
Dkk3	55.0%(11/20)	14.9%(11/74)	0.000	100.0%(20/20)	82.4%(61/74)	0.043

Discussion

Aberrant methylation of CpG islands in promoters had been ascertained as a primary mechanism for the inactivation of tumor suppressor genes in human malignancies [22]. The Wnt-antagonist genes, including *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3* genes, functionally acting as Wnt signaling inhibitors, were recently shown to be a common target of promoter hypermethylation in numerous tumor entities [8, 11, 13, 14]. In the present study, we found each of these six Wnt-antagonist genes had higher methylation levels and lower expression of mRNA transcripts in GCA tumor tissues compared with adjacent non-cancerous tissues. It had been suggested that the methylation of Wnt-antagonist genes may promote tumorigenesis. In addition, there was a significant concordance between promoter methylation of Wnt-antagonist genes and its lack of mRNA expression, and the inhibited frequencies of mRNA expression in complete methylation groups were significantly higher than those in incomplete methylation groups, which indicated that epigenetic silencing of Wnt-antagonist genes promoter via hypermethylation may be one of the critical mechanisms for inactivation of these genes in GCA. Urakami [8] and Nojima M et al. [18]. found that expression of *Wif-1* and *sFRPs* mRNA transcripts in bladder cancer cell lines and gastric cancer cell lines was significantly enhanced after methyltransferase inhibitor 5-Aza-dC treatment, confirming our results. In addition, we found that most of the Wnt-antagonists genes which methylated state were tumor specific, however, there were some cases in which the change of methylation was not only present in tumor tissues, but also in paired non-cancerous tissues. Given the high sensitivity of MSP analysis, it was possible that

Table 6 The frequency of Wnt-antagonist genes mRNA negative expression between complete methylation and incomplete methylation groups in GCA

gene	mRNA negative expression frequency		P
	complete methylation	incomplete methylation	
sFRP1	100.0%(19/19)	49.1%(27/55)	0.000
sFRP2	88.0%(22/25)	34.0%(16/47)	0.000
sFRP4	76.2%(16/21)	33.3%(15/45)	0.001
sFRP5	86.7%(26/30)	47.0%(20/43)	0.000
Wif-1	94.4%(17/18)	25.0%(10/40)	0.000
Dkk3	100.0%(7/7)	30.8%(4/13)	0.003

normal-appearing specimens contained few cancer cells which were undetectable by histomorphology, furthermore, the presence of the hypermethylation in corresponding non-cancerous tissues may represent the appearance of premalignant lesions [23]. In the study of Klump B, hypermethylation of the tumor suppressor gene p16 refering to neoplastic progression in Barrett's esophagus, was detected in pathologically normal-appearing specimens obtained from a patient who later developed dysplasia [24]. Therefore, epigenetic inactivation of tumor-associated genes may be an aberrant and early feature of tumorigenesis.

Our results showed that the inhibited frequencies of Wnt-antagonist genes mRNA expression in hypermethylation groups were significantly higher than those in unmethylation groups, however, minority samples also showed positive mRNA expression with genes methylation. In our studies, we found that the tumor tissues both showed hypermethylation and positive mRNA expression were all incomplete methylation. It is partly due to the fact that the partial promoter methylation was insufficient to suppress Wnt-antagonist genes transcription. Several studies had reported that density of CpG island methylation was related to the suppressed degree of transcription [25], weak promoter could be completely suppressed by lower density methylation, however, when promoter was enhanced by enhanser, function of transcription would be retrieved.

Although most of the digestive tract cancers showed hypermethylation of Wnt-antagonist genes, the frequency of methylation in these tumors and the correlation of methylation frequency with histological differentiation and tumor stage were different. Veeck et al. indicated that the promoter hypermethylation of *SFRP1* gene was significantly associated with tumour stage, but not with lymph node status, and histologic type of invasive breast cancer [26]. However, the study of Sogabe showed that *sFRP1,2,5* genes hypermethylation weren't connected with TNM classification and differentiation of tumors in oral squamous cell carcinoma [27]. Furthermore, Sato et al. demonstrated that *Dkk* genes family were frequent targets of epigenetic silencing in gastric carcinoma and colorectal cancer, and found that the methylation status of each *Dkk* gene was not statistically significant with the tumor pT status, pN status, pM status and tumor stage [17]. Here, we demonstrated that any Wnt-antagonist genes hypermethylation was not correlated with tumor stage and histological differentiation in patients with GCA. Different proportion of patients according to TNM classification and differentiation of tumors may be one of the reasons; type difference of tumors among these studies may also lead to the above reported difference. Furthermore, we found that the samples which showed simultaneous methylation of more than three genes had a remarkable correlation with tumor stage, suggesting that multiple gene methylation may have definite value on estimating prognosis of GCA patients.

ß-catenin is a key mediator of the canonical Wnt signaling pathway [7]. The accumulation of ß-catenin in cytoplasm /nuclear is a primary step required for the activation of the pathway and the target gene's hyperexpression, which induced by the ß-catenin/TCF/LEF transcriptional complex [28]. In our studies, we analyzed the relationship between Wnt-antagonists genes methylation status and the ectopic expression of ß-catenin in GCA. We found that ectopic expression of ß-catenin was significantly increased in GCA tissues compared with non-cancerous tissues, suggesting that Wnt signaling pathway maybe remains in active status in GCA. In addition, there was a remarkable concordance between promoter methylation of Wnt-antagonist genes and the ectopic expression of ß-catenin, which indicated that the hypermethylation of Wnt-antagonist genes may be one of the critical mechanism for a shift of ß-catenin protein from membrane to nucleus, which may through the aberrant canonical Wnt/ß-catenin signal activation involved in the pathogenesis of GCA. There are conflicting results regarding *Dkk3*. Sato et al. [17] showed that *Dkk3* did not affect the levels of β-catenin protein or inhibit β-catenin/TCF-induced transcription in prostate cancer cells. But Lee et al. [29] indicated that the forced expression of *Dkk3* markedly decreased the β-catenin levels in cervical cancer cell lines and cervical cancer tissue specimens. So they thought *Dkk3* was a negative regulator of β-catenin and its downregulation contribute to an activation of the β-catenin signaling pathway, which was similar with our study. Different kind of tumor may be one of the reasons that lead to the controversy, and the study environment was different between in vitro and in vivo experiment, which may be an important reason. Cyclin D1 had been identified as target genes of the Wnt signal in recent investigations [9, 10]. In this study, we found that CyclinD1 hyperexpression was correlated with all the Wnt-antagonist genes methylation in GCA. This strongly indicated that Wnt-antagonist genes were important human tumor suppressor genes which acted at the level of G1/S-phase cell cycle progression through the aberrant canonical Wnt/ß-catenin signal activation.

To our best knowledge, no study about Wnt-antagonist genes promoter methylation and mRNA expression in GCA had been reported, however, there were some studies about the correlation of promoter hypermethylation of Wnt-antagonist genes with gastric cancer and esophageal carcinoma [17-19]. In their studies, the hypermethylation of Wnt-antagonist genes were founded, and may contribute to the pathogenesis of gastric and esophageal cancer, which was consistent with our results. GCA, gastric and esophageal cancer were all originated from upper digestive tract, however several studies showed that the occurrence mechanism of these three tumors may be different, the similar methylation status of Wnt-antagonist genes in the three tumors indicated that the hypermethylation of Wnt-antagonist genes maybe produce a marked effect on upper digestive tract tumors through the Wnt signalling pathway.

In summary, our results suggested that the aberrant methylation of Wnt-antagonist genes were epigenetic events that silence these genes in gastric cardia adenocarcinoma. Further work is necessary to elucidate the exact function and interaction with other factors to develop strategies for early diagnosis, prevention and treatment of gastric cardia adenocarcinoma.

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