

Hypermethylation of multiple Wnt antagonist genes in gastric neoplasia

Is *H pylori* infection blasting fuse?

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Abstract

Wnt antagonist genes hypermethylation has been found in several tumors. Accordingly, the events that occur during the progression of adenoma to carcinoma have been characterized and include activation of the Wnt-pathway. Further, gastric adenoma (GA) is a premalignant lesion of gastric adenocarcinoma (GAC). In this paper, we focused our interesting on Wnt signaling path function in the pathogenesis of GAC.

We compared the differences between low grade adenoma (LGA), high grade adenoma (HGA), GACs and corresponding normal gastric tissue (NGT). Specific indexes include the pathological characteristics of gastric neoplasia, *Helicobacter pylori* infection, β -catenin mutation status, and methylation status of Wnt antagonist genes.

There was significant difference of β -catenin expression in patient with NGT, LGA, HGA, and GAC, the results respectively were 4.2%, 41.7%, 83.3%, and 91.7%. Only 1 GACs was detected exon 3 of β -catenin mutation. Wnt antagonist genes mRNA expression levels, such as *APC*, *sFRP-1*, *Wif-1*, and *Dkk-1*, were significantly reduced in GAC. Promoter methylation levels of the 4 genes were significantly elevated in GAC and HGA compared to NGT and LGA. However, there was no significant difference between HGAs and GACs. The β -catenin abnormal expression was correlated with hypermethylation of these 4 genes. Multiple gene concurrent methylation phenomenon was increased from NGTs to GACs; the amount of methylation genes in GACs and HGAs was more than NGTs and LGAs. The more methylation of the above-mentioned genes, the more severity of local inflammation. The infection rate of *H pylori* was significantly higher in patient with HGA (66.7%, 16/24) and GAC (58.5%, 14/24) than in LGAs (16.7%, 4/24) ($P_{\text{HGA-LGA}} = .024$, $P_{\text{GAC-LGA}} = .032$). In addition, the present of *H pylori* also correlated with the β -catenin abnormal expression and the hypermethylation status of Wnt antagonist genes ($P < .001$). But other parameters in adenoma cases had no significantly related with infection of *H pylori*.

Hypermethylation of Wnt antagonist genes may have a tight relationship with gastric tumorigenesis. And these genes may increase the incidence of GAC. Additionally, *H pylori* may have promotion function in GA formation.

Abbreviations: DKK = DICKKOPF, GA = gastric adenoma, GAC = gastric adenocarcinoma, HGA = high-grade adenoma, IRS = immunoreactive score, LGA = low-grade adenoma, MSP = methylation-specific PCR, NGT = normal gastric tissue, *H Pylori* = *Helicobacter pylori*, sFRP = secreted Frizzled-related proteins, TCF/LEF = T cell factor/lymphoid enhancer factor, USP = unmethylation-specific PCR, WIF-1 = Wnt inhibitory factor-1.

Keywords: gastric neoplasia, *H pylori*, hypermethylation, Wnt antagonist gene

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1. Introduction

Currently, there was a common view that gastric cancer is one of the most common and deadly cancers in the worldwide. It is important to study early etiology and detection to develop effective methods for the diagnosis of fatal diseases. Gastric adenocarcinoma (GAC) usually progressed as the order of inflammation, metaplasia, dysplasia, and cancer.^[1] There were 2 high risks of GACs: intestinal metaplasia (IM) and gastric epithelial dysplasia (GED), that was to say, neoplastic precancerous lesions. A unique neoplastic growth characteristic of gastric adenoma (GA) is dysplastic epithelium localized polypoid proliferation. This growth characteristic tends to progress to infiltrating adenocarcinoma.^[2] The current opinion considered that genetic epigenetic abnormalities may be involved in specific pre-malignant lesions. Its significance was not just confined to innocent atrophic gastritis.^[3] Therefore, it helps to understand the progress from GA to GAC and identify pathogenic genes.

Literature has now proved that the key of adenomas to cancer was suppressor *TP53* losses, as well as *KRAS* and Wnt pathway

activation.^[4] The Wnt signaling pathway plays an important role in tumorigenesis.^[5] The classical Wnt signal pathway activation promoted the transcription of several target genes after the interaction of β -catenin accumulation and T-cell factor/lymph enhancer factor (*TCF/LEF*).^[6] Various other human malignancies had been proved they are conducted by the aberrant activation of Wnt signaling way.^[5,7-9]

Currently, we had found that the development and progression of GA were facilitated by methylation of the *APC* promoter rather than mutations of *APC* and β -catenin. Wnt antagonist genes such as *APC*, *AXIN-2*, Frizzled-related proteins (*sFRPs*), Wnt inhibitor (*WIF-1*) and *DICKKOPFs* (*DKKs*) in cancer may play an important role in the stabilization and accumulation of β -catenin. The methylation of the Wnt signaling pathway is moderately activated in physiological status.^[10] If there are aberrant methylation of above Wnt antagonist genes, Wnt/ β -catenin signal way will be activated.

Recently, the widely accepted view was that *Helicobacter pylori* (*H pylori*) infection have a close relation with gastric cancer (GC). *H pylori* contributes to epigenetic changes is known to increase the risk of GC in the future.^[11] In addition, some studies proved that E-cadherin gene, p16 gene, and *APC* gene methylation had closely correlation with *H pylori* infection. However, the bacterial role in GA development and progression is not clear.

Genes methylation was important in GAC development because many genes have changed methylation patterns in the tumor. In this study, we evaluated

1. the changes about Wnt antagonist genes methylation status from GA to GAC,
2. the pathological characteristics correlation between gastric neoplasia, β -catenin mutational status, and Wnt antagonist genes expression and methylation status. And
3. the correlation between *H pylori* and the hypermethylation status of Wnt antagonist genes.

2. Methods

2.1. Specimens

Ethics committee of Jinling Hospital approved this study. There were 24 samples of primary GACs and corresponding normal gastric tissues (NGTs) in this study. And all the samples were obtained by radical or partial gastrectomy. The 48 cases which proceeding gastroscopic polypectomy were included. Among them, 24 cases were low-grade adenomas (LGAs) and other cases were high-grade adenomas (HGAs). Corresponding periadenomatous NGTs were obtained by endoscopic gastric biopsies. Histological analysis of selected biopsy materials showed that these samples contained 40% to 80% of epithelial tissues. Vienna Classification was the criterion for sample classification in this study.^[12] The updated Sydney System in periadenomatous NGTs was a standard for the assessment of inflammation, glandular atrophy, and intestinal metaplasia.^[13] First, the sample was immobilization by 10% buffered formalin (pH 7.0). Then the sample was embedded in paraffin wax. Sections (5 μ m) of the sample was stained by hematoxylin and eosin for evaluation of histology. Except analysis, samples were stored at -80°C to ensure stability.

2.2. Immunohistochemistry

Immunohistochemical stain with anti- β -catenin (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using a 5 μ m

thick section of formalin fixed and paraffin embedded (FFPE) sections and incubated 2h at room temperature. Avidin-biotin-peroxidase complex procedure (ABC standard; Vector Laboratories, Burlingame, CA, USA) was performed. Peroxidase activity was measured using 3,3'-diaminobenzidine tetrachloride as a substrate.

Cytoplasm and cell membrane were mostly possible expressed place of β -catenin. β -catenin immunoreactive score (IRS) was a widely used semiquantitative compiled method. We used different scores to represent β -catenin staining intensity: if the score was 0, represented no staining; if the score was 1, represented weak staining; if the score was 2, represented moderate staining; and if the score was 3, represented strong staining. The positivity area evaluated by values. 0 represented focal or <10%, 1 represented 10% to 30%, 2 represented 30% to 50% and 3 represented >50%. The final IRS score was added score of staining intensity and score of percentage of positivity, the IRS score range from 0 to 6. The IRS pattern was divided into "weak" (IRS score <3) or "strong" (IRS score >3). Only when experiment result of immunohistochemical test showed strong membrane staining and weak cytoplasmic staining in the meantime, mean "normal" pattern. Except the only pattern, others all represented the "disordered" pattern. β -catenin immunostaining was evaluated by 2 independent observers which were blinded to patients' clinical inspection results and local staging.

2.3. DNA and RNA isolation

In this study, TRIzol Reagent (Life Technologies, Breda, The Netherlands) was used to isolate DNA and RNA from cell lines.^[14] DNA from the FFPE material was isolated after the previously mentioned macro-dissection.^[15]

2.4. Analysis of the β -catenin gene

For β -catenin, genomic polymerase chain reaction (PCR) technology was used. Exon 3, a fragment previously thought to be activated by mutations, was amplified as method previously reported in the literature.^[16]

High-fidelity Primestart DNA polymerase (HotStart version; TaKaRa, Dalian, China) was used in PCR procedure. Sequencing procedure was performed using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). To avoid mistakes, the above operation was repeated once more for the mutated sample.

2.5. Reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) analysis

RT-PCR analysis of *APC*, *sFRP-1*, *DKK-1*, and *WIF-1* expression was performed using cDNA synthesized from 1 μ g of total RNA. 2% agarose gel was used to analyze the PCR products. qRT-PCR was performed using an iCycler with iQ SYBR Green Supermix (Bio-Rad) and the same gene-specific primers. The internal standard was β -actin.^[17] The relative amount of the test report was calculated by the formula $2^{-\Delta\Delta\text{Ct}}$, where ΔCt was the representative mean subtraction of β -actin Ct from the mean target Ct value.

2.6. Methylation-specific PCR (MSP)

Commercially available sodium bisulfite (Invitrogen, Carlsbad, CA, USA) was used to modify genomic DNA. *APC*, *sFRP-1*,

DKK-1, and *WIF-1* were the targeted genes of this study. The first universal primer does not contain forward or reverse CpG site primers, and the amplified DNA fragment contains multiple promoter regions. The second round of nested MSP or unmethylated specific PCR (USP) was the use of a universal PCR product template. Primer sequences designed for MSP and USP Wnt antagonist genes have been reported.^[18] Preliminary tissue MSP semi-quantitative analysis of each primer PCR reaction cycle linear range. The PCR mixture was separated by 1.5% agarose gel electrophoresis. The gel contained ethidium bromide. For the conjugated structure of DNA bonds, PCR products were determined by UV light. For the specimen has a positive MSP bond, nondenaturing 12% polyacrylamide gel electrophoresis was used to separate MSP or USP and then determined relative methylation ratio. Image J software was used to calculate the corresponding each band area under the curve (AUC).^[4] Previously reported studies have determined the degree of correlated methylation [MSP ratio = MSP band density / (MSP band density + USP band density)].^[19,20]

2.7. Genetic analysis of *H pylori*.

The presence of the *glmM* gene was confirmed in DNA samples extracted from PT and GA by semi-nested PCR and sequencing as described above.^[21] Sequence analysis of the PCR products was performed using an ABI 310 Genetic Analyzer (Applied Biosystems). DNA base transformation was always made independently.

2.8. Statistical analysis

The result was described by statistical parameters mean and standard deviation (SD). And the final results were summarize by and bar charts. *T* test and Welch method (2-sided) was used to determine the distinguish of expression levels. Categorical variables were analyzed by χ^2 or Fisher exact tests. Binomial logistic regression analysis was used to subordinately analyze categorical variables multivariate. $P < .05$ mean there existed statistically significant. All data were analyzed using SPSS software for Windows 13.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Clinical and histological characteristics

Table 1 shows the patient's clinical and histological features. Thirty men and 18 women participated in this study with an average age of 56 years (range: 23–82 years). The average size of adenomas is 9.4 mm, ranging from 6 to 25 mm, with adenomas more common than flattened glands. Most of the cases less than 10 mm. All periadenomatous NGTs divided into various grades of inflammation. Among the cases, 5 accompanied intestinal metaplasia while 3 accompanied glandular atrophy. The inflammation grade showed a strong correlation with the size of GAs ($P = .000$) and grade ($P = .006$).

3.2. Immunohistochemistry for β -catenin expression in different gastric epithelial tissues

To test Wnt/ β -catenin signaling status in different gastric epithelial tissues, we firstly evaluated the localization of β -catenin in clinical tissues. In most NGT patients, β -catenin was mainly found in cell membranes and partly in cytoplasm. For LGAs, 14 of the 24 cases

Table 1

Clinicopathological characteristics of the 48 GAs.

Variables	Total cases (n=48) n (%)	Grade of adenomas	
		Low (n=24) n (%)	High (n=24) n (%)
Gender distribution			
Male	30 (62.5)	14 (58.3)	16 (66.7)
Female	18 (37.5)	10 (41.7)	8 (33.3)
Age (years)			
<50	17 (35.4)	9 (37.5)	8 (33.3)
≥50	31 (64.6)	15 (62.5)	16 (66.7)
Growth pattern			
Flat	13 (27.1)	6 (25)	7 (29.2)
Polypoid	35 (72.9)	18 (75)	17 (70.8)
Size (mm)			
<10	27 (56.3)	18 (75)	9 (37.5)
≥10	21 (43.7)	6 (25)	15 (62.5)
Histology of NGTs			
Inflammation			
Mild	24 (50)	16 (66.7)	8 (33.3)
Moderate	16 (33.3)	5 (20.8)	11 (45.9)
Severe	8 (16.7)	3 (12.5)	5 (20.8)
Glandular atrophy	3 (6.25)	2 (8.3)	1 (4.2)
Intestinal metaplasia	5 (10.4)	3 (12.5)	2 (8.3)

GA = gastric adenomas, NGT = corresponding normal gastric tissues.

(58.3%), β -catenin mainly existed in the cell membrane, but the rest cases (41.7%) were acted as a disordered pattern. The β -catenin expression of these 10 cases was quite different from NGTs which expressed strongly in the cytoplasm. In the case of HGAs, β -catenin was found weakly in the cell membrane and strongly expressed in cytoplasm (20/24, 83.3%). Sometimes, weak nuclear staining could be observed. For the gastric cancer cases, β -catenin was found strong expression in cytoplasmic or nuclear rather than the cell membrane. Nearly all the inclusion cases could found this phenomenon (22/24, 91.7%) (Fig. 1). The difference of β -catenin expression between GACs and NGTs was statistically significant ($P < .005$) (Table 2). However, the statistical differences did not exist between HGAs and GACs.

3.3. Mutation in exon 3 of β -catenin

None of the included cases of LGAs, HGAs, and NGTs was found mutations in exon 3 of β -catenin. Only 1 gene mutation was observed for 24 GACs (4.2%). By sequencing analysis, mutations in the in-frame 6 bp deletions (delGGTGCC, Gly38Ala39) were found at codons 38 and 39.

3.4. mRNA expression of Wnt antagonist genes

The negative regulators that inhibit the Wnt/ β -catenin signaling pathway, such as *APC*, *DKK-1*, *sFRP-1*, and *WIF-1*, were detected by qRT-PCR. Wnt antagonist gene expression decreased in HGAs and GACs (Fig. 2A). To confirm these results, qRT-PCR was also used to study mRNA expression levels. We also found that mRNA expression levels of GACs were significantly lower than LGAs ($P = .000$) (Fig. 2B).

3.5. MSP for Wnt antagonist genes

In order to clarify whether the reduction of expression of the Wnt/ β -catenin signaling pathway antagonist mentioned above was

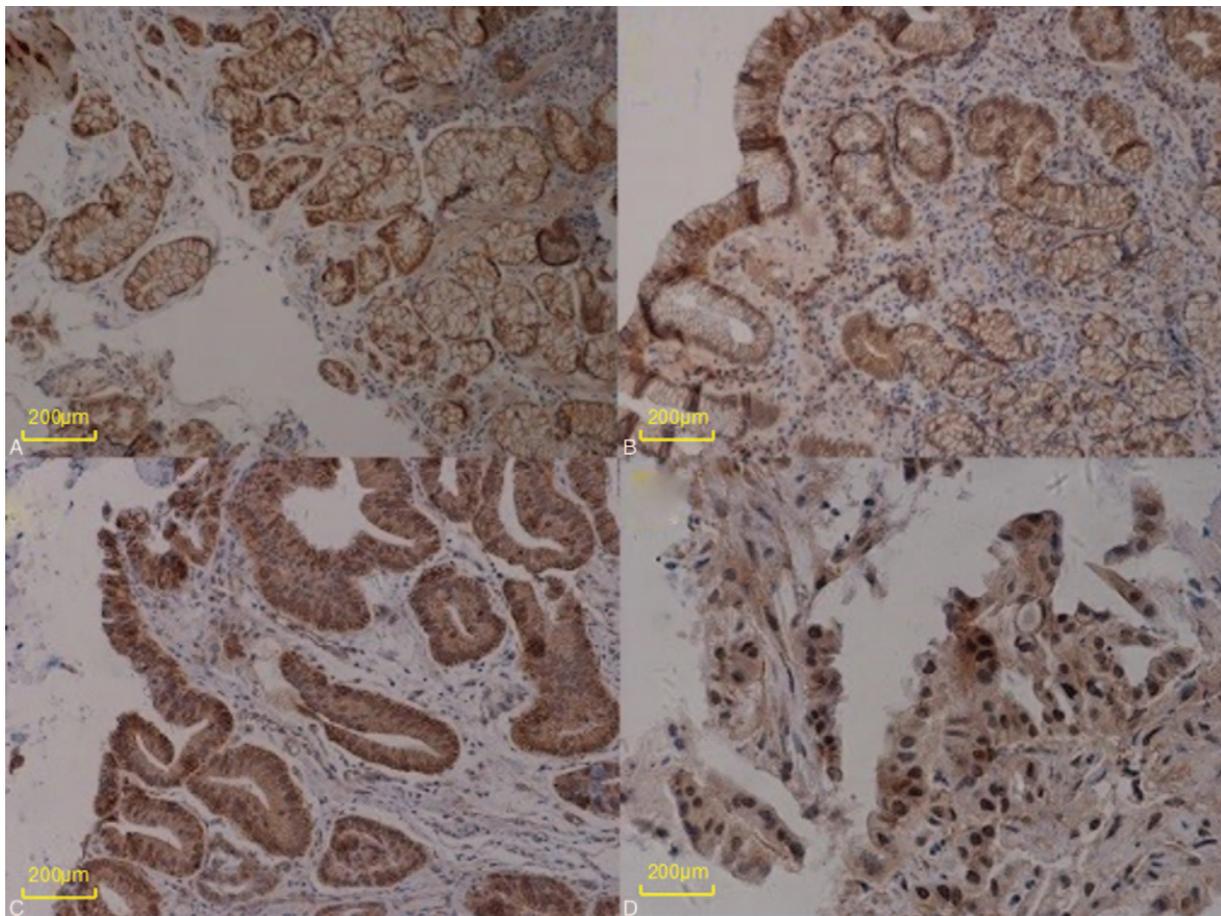


Figure 1. β -Catenin expression in NGTs, GAs and GCs. (A) NGTs immunostained for β -catenin. Note the presence of strong membrane staining and weak cytoplasmic staining. Hematoxylin counterstain; original magnification: $\times 200$. (B) LGAs immunostained for β -catenin. Note the presence of moderate membrane staining in major cells and weak cytoplasmic staining. Hematoxylin counterstain; original magnification: $\times 200$. (C) HGAs immunostained for β -catenin. Note the presence of strong cytoplasmic staining and mild nuclear immunoreactivity. Hematoxylin counterstain; original magnification: $\times 200$. (D) GCs immunostained for β -catenin. Note the presence of strong cytoplasmic staining and nuclear immunoreactivity. Hematoxylin counterstain; original magnification: $\times 400$. GA=gastric adenoma, GC=gastric adenocarcinoma, HGA=high-grade adenoma, LGA=low-grade adenoma, NGT=normal gastric tissues.

due to the methylation of CpG islands, we used MSP analysis to detect the methylation status of the CpG islands. The methylation levels of *APC*, *DKK-1*, *sFRP-1*, and *WIF-1* promoters in GAC and HGA were significantly increased compared to NGTs and LGAs (Table 3). For *APC* promoter methylation levels, there was no significant difference between NGTs and LGAs ($P = .252$). For above genes' promoter methylation levels, there were no

Table 2
 β -catenin expression in different gastric epithelial tissues, n (%).

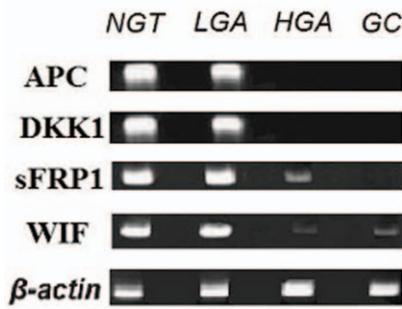
Type of tissues	Cases	Normal	Abnormal
NGTs	72	69 (95.8)	3 (4.2)
GA	48	18 (37.5)	30 (62.5)
LGAs	24	14 (58.3)	10 (41.7)
HGAs	24	4 (16.7)	20 (83.3)
GACs	24	2 (8.3)	22 (91.7)

HGAs versus LGAs, $P = .002$. LGAs versus NGTs, $P < .001$. HGAs versus NGTs, $P < 0.001$. GACs versus NGTs, $P < .001$. GACs versus LGAs, $P < .001$. GACs versus HGAs, $P = .394$. GAC=primary gastric adenocarcinoma, HGA=high-grade adenoma, LGA=low-grade adenoma, NGT=corresponding normal gastric tissues.

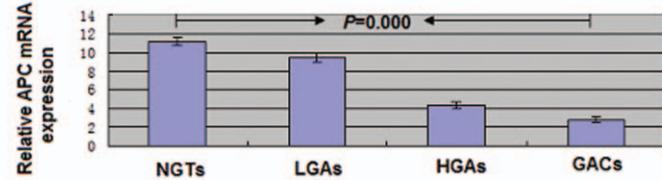
significant differences between HGAs and GACs ($P_{APC}=1.0$, $P_{DKK1}=.568$, $P_{sFRP1}=.561$ and $P_{WIF1}=.251$). Promoter methylation of the 4 genes related with aberrant expression of β -catenin (Table 4). There were more concurrently genes methylated increased from NGTs to GACs. GACs and HGAs had more concurrently methylated genes in GACs and HGAs than NGTs and LGAs ($P = .000$) (Fig. 3). About the number of simultaneous methylation genes, there was no significant difference between GACs and HGAs ($P = .284$), or between NGTs and LGAs ($P = .162$). However, there was a significant difference in the number of simultaneously methylated genes between LGAs and HGAs ($P = .000$). The methylation level of these genes is correlated with the level of local inflammation ($P = .000$, $r = .287$).

3.6. Presence of *H pylori* and correlation of the *H pylori* status and various factors

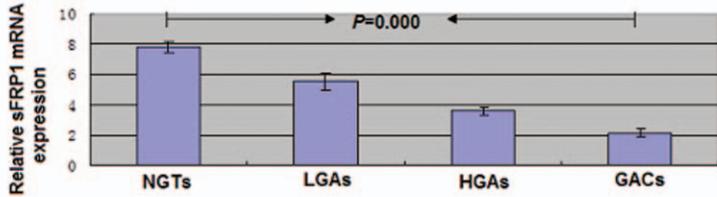
In this study, in addition to C^{13} respiration test, PCR were used to measure the *H pylori glmM* gene sequence as an indicator of the degree of *H pylori* infection. *H pylori* was detected in 37.5% (27/72) by C^{13} respiration test, 23.6% (17/72) by PCR, and 47.2%



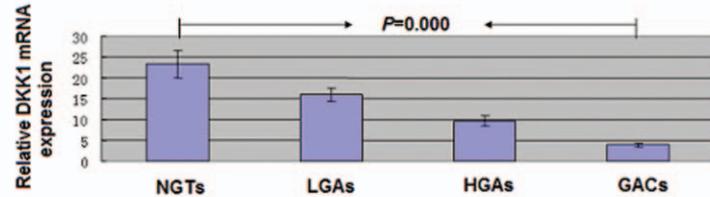
Expression analysis of Wnt/β-catenin inhibitor genes in different gastric tissues



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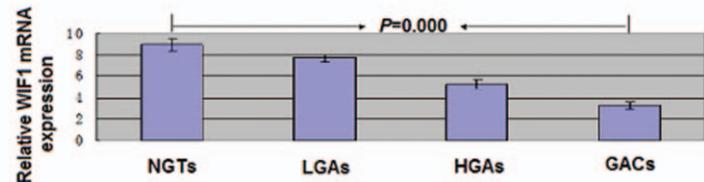


Figure 2. Expression analysis of Wnt/β-catenin inhibitor genes in different gastric epithelial tissues. (A) RT-PCR analysis of APC, DKK-1, sFRP-1, and WIF-1 expression in different gastric epithelial tissues. Expression of Wnt inhibitor genes was weaker in HGAs and GCs. (B) qRT-PCR analysis confirmed that mRNA expression levels of the examined genes were significantly decreased from LGAs to GCs ($P = .000$). The relative amount of mRNA expression was calculated by the comparative $\Delta\Delta C_t$ method. β-Actin was used as internal control in both analyses. GC=gastric adenocarcinoma, HGA=high-grade adenoma, LGA=low-grade adenoma, NGT=normal gastric tissue.

(34/72) by combining the 2 test results. The experiment result showed that the infection rate was significantly higher in HGAs (66.7%, 16/24) and GAC (58.5%, 14/24) than in LGAs (16.7%, 4/24) ($P_{HGA-LGA} = .024$, $P_{GAC-LGA} = .032$), and there were similar

features in β-catenin abnormal expression cases and Wnt antagonist gene hypermethylation cases. However, no other parameters of adenoma cases associated with *H pylori* infection were found in this study.

Table 3

Frequency of promoter methylation of the Wnt antagonist genes in different gastric epithelial tissues.

	NGTs (%)			Total	LGAs	HGAs	GACs
	LGAs	HGAs	GCs				
APC	1/24 (4.2)	2/24 (8.3)	3/24 (12.5)	6/72 (8.3)	3/24 (12.5)	19/24 (79.2)	20/24 (83.3)
DKK1	0	0	0	0	2/24 (8.3)	9/24 (37.5)	11/24 (45.8)
sFRP1	0	0	0	0	2/24 (8.3)	14/24 (58.3)	16/24 (66.7)
WIF	0	0	0	0	2/24 (8.3)	12/24 (50.0)	15/24 (62.5)

NGTs:LGAs, $P_{APC} = 0.252$, $P_{DKK1} = P_{sFRP1} = P_{WIF1} = 0.013$.

HGAs:GACs, $P_{APC} = 1.0$, $P_{DKK1} = 0.568$, $P_{sFRP1} = 0.561$, $P_{WIF1} = 0.251$.

NGTs:HGAs and NGTs:GACs, $P_{APC} = P_{DKK1} = P_{sFRP1} = P_{WIF1} = 0.000$.

LGAs:GACs, $P_{APC} = P_{sFRP1} = P_{WIF1} = 0.000$, $P_{DKK1} = 0.003$.

LGAs:HGAs, $P_{APC} = P_{sFRP1} = 0.000$, $P_{DKK1} = 0.016$, $P_{WIF1} = 0.001$.

GAC=primary gastric adenocarcinoma, HGA=high-grade adenoma, LGA=low-grade adenoma, NGT=corresponding normal gastric tissues.

Table 4

Correlation between status of the Wnt antagonist genes methylation and immunohistochemical expression of β -catenin in different gastric epithelial tissues.

Genes	Methylation status	n	IM of β -catenin		P value	r
			Normal	Abnormal		
APC	ME	50	4	46	0	0.808
	UM	94	85	9		
DKK1	ME	22	0	22	0	0.54
	UM	122	89	33		
sFRP1	ME	32	5	27	0	0.508
	UM	112	84	28		
WIF1	ME	30	1	29	0	0.616
	UM	114	88	26		

ME = methylated, UM = unmethylated.

4. Discussion

β -catenin played an important role in classical Wnt signaling pathway.^[22] In the cytoplasm and nucleus, β -catenin/TCF/LEF transcription complex is formed by accumulated β -catenin and induces over-expression of the target gene.^[23] In cases of NGTs, LGAs, HGAs and GACs, we detected ectopic expression rate of β -catenin with the result of 4.2%, 41.7%, 83.8%, and 91.7%, respectively. The difference between 3 gastric tumor tissues and NGT was significant ($P < .001$). However, there was no significant difference between HGAs and GAC ($P = .682$). The abnormal expressions of Wnt/ β -catenin in GAs and GAC were similar because the histological characteristics of HGA were closer to GACs than LGAs.

Recent Wnt signaling pathways were research hotspots and several antagonists of this pathway had been identified. APC, sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1, and Dkk-3 acted as Wnt antagonist genes by binding Wnt molecules or low-density lipoprotein receptor related protein LRP5/LRP6 components receptor complex. Therefore, if Wnt antagonists losing its function, the Wnt pathway could be activated and induced ectopic expression of β -catenin. So far, Wnt antagonist genes

downregulation not only find in GAC, but also in other malignancies, including bladder,^[18,24,25] lung^[26,27] and breast^[28] cancer, chronic lymphocytic leukemia,^[29] and even esophageal^[30] carcinoma. In our study, mRNA expression levels of the examined Wnt-antagonist genes were significant lower in GACs than LGAs ($P = .000$). The correlation between Wnt antagonist gene methylation status and β -catenin ectopic expression was analyzed in this study for 4 gastric tissues. Compared to NGTs and LGAs, APC, DKK-1, sFRP-1, and WIF-1 promoter methylation levels were significantly elevated in GACs and HGAs ($P < .001$). In addition, for APC, DKK-1, sFRP-1, and WIF-1 promoter methylation levels, there were no significant differences between HGAs and GACs ($P_{APC} = 1.0$, $P_{DKK1} = .568$, $P_{sFRP1} = .561$, and $P_{WIF1} = .251$). Wnt antagonist genes promoter methylation and β -catenin ectopic expression existed remarkable consistency. This indicated that hypermethylation of the Wnt antagonist gene was one of the key mechanisms of transferring the β -catenin protein from the cell membrane to the nucleus. This may be mediated through the abnormal Wnt/ β -catenin signal activation involved in the pathogenesis of GAs and GACs. The occurrence rate of genes concurrently methylated increased from

The numbers of methylated genes in different gastric tissues

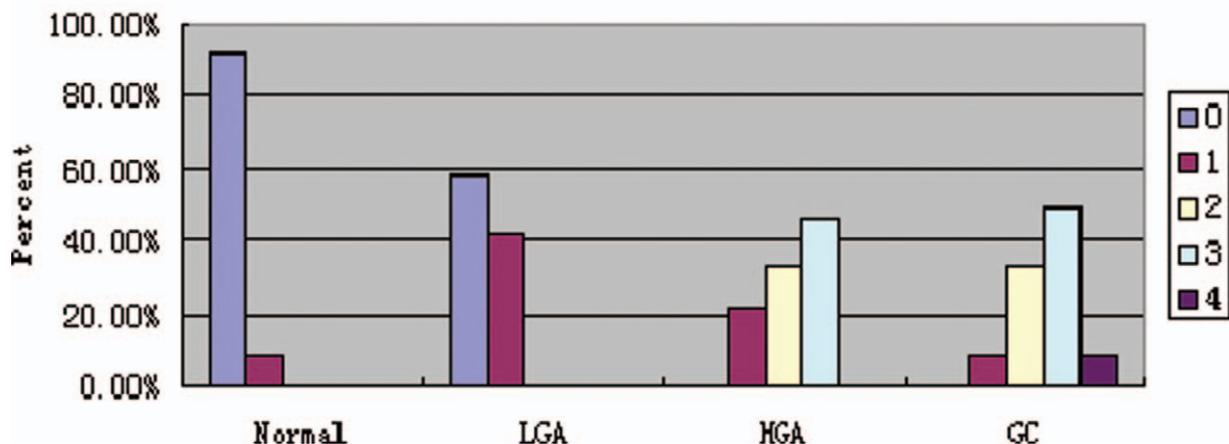


Figure 3. Summary of the methylation profile in NGTs, LGAs, HGAs and GACs. Concurrently methylated genes increased from NGTs to GACs. GACs and HGAs had more concurrently methylated genes than NGTs and LGAs ($P = .000$). There was no significant difference in the number of concurrently methylated genes between GACs and HGAs ($P = .284$) or between NGTs and LGAs ($P = .162$). GAC = gastric adenocarcinoma; HGA = high-grade adenoma; LGA = low-grade adenoma; NGT = normal gastric tissue.

NGTs to GACs. Occurrence rate of concurrently methylated genes of GACs and HGAs were higher than NGTs and LGAs ($P=.000$). This finding may reflect that aberrant methylation of Wnt antagonist genes is a sign of precancerous lesions or early stage of gastric cancer and these genes involve in early initiation and transformation process.

In addition, the study reported by Guo et al^[31] indicated that most of the Wnt antagonist genes were methylated in some specific tumors. However, in some cases, methylation changes exist in tumor tissue as well as paired non-cancerous tissues. For the high sensitivity of MSP analysis, though para-tumor tissues could not find the character of cancer cell in the histomorphology, some genes hypermethylation presented to these samples or the premalignant lesions.^[30] In the current study, the *APC* gene methylation in NGTs was found, it is quite different from other 3 Wnt antagonist genes. There did not exist significant difference between NGTs and LGAs ($P=.252$). Klump et al^[32] found that tumor suppressor gene p16 hypermethylation showed Barrett's esophageal tumor progression, normal pathology later developed into dysplasia. Thus, *APC* genes epigenetic inactivation might be an early signal of tumorigenesis in GACs.

As far as we know, no studies on the promoter methylation and mRNA expression of Wnt antagonist genes in GA had been made so far. But the relationship between Wnt antagonist genes promoter hypermethylation and gastric cancer/ esophageal carcinoma studied by some researcher.^[30,33,34] Results of Yoshida and Saito^[35] indicated that thirty percent of the GAs resected by endoscopy were associated with canceration, which suggested that the underlying pathogenesis of adenomas was similar to that of cancer. Another GAs study showed the risk of development to GCs was about 2.5% to 50%.^[36,37] According to a recent opinion, GA is a more likely developed to cancer than atrophic gastritis alone.^[3] Thus, we assess the relationship between the four Wnt antagonist genes of promoter hypermethylation, expression and mutation of β -catenin, and histologic features in GAs. The β -catenin aberrant expression exists in the NGTs, LGAs, and HGAs and the detected rate was 4.2%, 41.7%, and 83.3%, respectively ($P<.001$). Mutations of β -catenin exon 3 in LGA and GA were not detected. Compared with *LGA*, *APC*, *DKK-1*, *sFRP-1*, and *WIF-1* promoter methylation levels were significantly increased in HGA ($P<.05$). There was a significant difference in the number of methylated genes between LGAs and HGAs ($P=.000$). Above mentioned genes methylation levels had a correlation with local inflammation level ($P=.000$, $r=.287$). The results indicated that gastric inflammation could be involved in the occurrence and development of GAs. This phenomenon also implies GA was a particularly premalignant lesion unlike glandular atrophy and intestinal epithelization.

CpG island promoter region hypermethylation is an epigenetic event. Several factors were found to cause this result, such as nitrosamines, tobacco tar, alcohol, high salt intake, and bacterial overgrowth. Especially *H pylori* infection can cause hypermethylation of some tumor suppressor genes. The researchers came from Japan have shown that GAs are associated with *H pylori* infection.^[38] Study result of Komoto et al^[39] also showed that GAs often accompanied with *H pylori* infection. Now there was much evidence showed that *H pylori* infection was significantly associated with methylation of E-cadherin, p16 and *APC* genes. Additionally, eradication of *H pylori* reversed the methylation of these genes in patients with chronic gastritis.^[40] *H pylori* of HGA patients (66.7%, 16/24) was significantly higher than that of LGAs (16.7%, 4/24) ($P=.024$) as compared with

previous studies. Similar result was found between LGAs and GACs. We did not find statistically significant correlations with adenoma growth pattern, size and gene mutation, but there was significant correlation with hypermethylation and expression of every Wnt antagonist genes. Therefore, we believe that *H pylori* plays a stimulatory role in GAs.

There are some drawbacks of this study, the result was based on small sample of inclusion patients and cancer-related genes. Thus, our results need larger multi-gene studies to prove. The exact function and interaction with other factors need more further work to elucidate. On the basement of further study, early diagnosis, prevention, and treatment strategies would be developed for GAC. And it was worth to further study the mechanism about *H pylori* how to influence hypermethylation of multiple genes.

In conclusion, our data suggest that hypermethylation of various Wnt antagonist genes had a close relationship with irregular activation of Wnt signaling and finally caused gastric tumor. Thus, the methylation status of the Wnt antagonist gene plays an important mediator role in the progression of gastric cancer and may improve objective criteria, such as endoscopic mucosal resection. In this regard, hypermethylation testing can evolve as a diagnostic tool to determine the risk of histological progression of the GAC.

Author contributions

Author contributions: Wang ZK wrote the paper; Ye YQ designed and analyzed; Dan Liu collected the specimen; Xiaoqian Yang reviewed histopathology of specimen; Wang FY checked the article.

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