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Research Article

Hypermethylation of Multiple Wnt Antagonist Genes in Gastric Neoplasia: Novel Mechanism of Gastric Tumorigenesis

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Abstract

Background and Aim: Hypermethylation of Wnt antagonist genes has been found in several cancers. Gastric adenoma is a premalignant lesion of gastric adenocarcinoma (GAC). In the present study, we aimed to determine how Wnt signaling plays a role in the pathogenesis of GAC.

Methods: We investigated the relationship among the pathological characteristics of gastric neoplasia, β-catenin mutational status, and expression and methylation status of Wnt antagonist genes by comparing low-grade adenomas (LGAs), high-grade adenomas (HGAs), GACs and corresponding normal gastric tissues (NGTs).

Results: Abnormal expression of β-catenin in NGTs, LGAs, HGAs and GACs was 4.2%, 41.7%, 83.3% and 91.7%, respectively. Only one was detected exon 3 of β-catenin mutation in GACs. The mRNA expression levels of Wnt antagonist genes, including APC, sFRP-1, Wif-1 and Dkk-1, were significant decreased in GACs compared to LGAs. Compared to NGTs and LGAs, promoter methylation levels of the four genes were significantly elevated in GACs and HGAs. However, there was no significant difference between HGAs and GACs. Methylation of the four genes correlated with abnormal expression of β-catenin. The number of concurrently methylated genes increased from NGTs to GACs; GACs and HGAs had more concurrently methylated genes than NGTs and LGAs. The levels of methylation of the above genes correlated with the degree of local inflammation.

Conclusions: Hypermethylation of Wnt antagonist genes may play an important role in gastric tumorigenesis, and could be an attractive target for management of gastric neoplasia at risk of progression to cancer.

Abbreviations

GA =Gastric adenoma, GAC=gastric adenocarcinoma, LGAs=low-grade adenomas, HGAs=high-grade adenomas, NGTs=normal gastric tissues, IM=Intestinal metaplasia, GED=gastric epithelial dysplasia, sFRPs=secreted Frizzled-related proteins, WIF-1= Wnt inhibitory factor-1, DKKs=DICKKOPFs, TCF/LEF=T cell factor/lymphoid enhancer factor, RT-PCR=Reverse transciption-PCR, qRT-PCR=quantitative reverse transcription-PCR

Introduction

Gastric cancer is still regarded as one of the most frequent and lethal types of cancer worldwide. To prevent the disease, an understanding of the etiological factors and the mechanisms in its early phase is required. Gastric adenocarcinoma (GAC), in most cases, represents the culmination of an inflammation–metaplasia–dysplasia–carcinoma sequence [1]. Intestinal metaplasia (IM) and gastric epithelial dysplasia (GED) lesions, which confer a high risk for the development of gastric cancer, are neoplastic precancerous lesions. Gastric adenoma (GA) is a rare neoplastic growth that is characterized by localized polypoid proliferation of dysplastic epithelium that tends to progress to infiltrating adenocarcinoma and is associated with a high risk of adenocarcinoma elsewhere in the stomach [2]. GA is considered a more specific premalignant lesion than atrophic gastritis alone [3]. Therefore, it is helpful to understand the progression from GA to GAC and to identify the genes responsible for its onset.

Established events during the progression of adenoma to carcinoma are the loss of tumor suppressor TP53, and constitutive activation of KRAS and the Wnt pathway [4]. The Wnt signaling pathway plays an important role in tumorigenesis and tumor development [5]. Canonical Wnt signaling activation is involved in the accumulation of β-catenin by inhibiting its degradation, and promotes the transcription of several target genes through interaction with the T cell factor/lymphoid enhancer factor (TCF/LEF) [6]. Aberrant activation of Wnt signaling has been reported in various human malignancies [5,7–9]. Recently, we found that hypermethylation of APC promoter, instead of mutations involving APC and β-catenin, may play a role in the development and progression of GA, contributing to moderate activation of Wnt signaling [10]. However, methylation of various genes has been observed in many cases of GAC. Next to activation of the Wnt signaling pathway via inactivation of the APC gene (e.g. by mutation, deletion or hypermethylation), methylation- mediated silencing of other upstream Wnt signal-regulating genes may present an alternative mechanism of constitutive Wnt pathway activation in GAC [11,12].

Wnt antagonists, including members of the destruction complex for β-catenin (APC and Axin-2), are commonly mutated and demonstrate a high frequency of aberrant promoter methylation [13–16]. In addition, epigenetic silencing of extracellular Wnt antagonists, such as secreted Frizzled-related proteins (sFRPs), Wnt inhibitory factor (WIF)-1 and DICKKOPFs (DKKs) may contribute to the stabilization and accumulation of β-catenin in cancers, with or without mutational activation of Wnt/β-catenin signaling.[17–21]. Methylation plays an important role in GAC development and many genes have altered methylation patterns in the tumor compared to normal gastric mucosa. Therefore, we assessed (1) how the methylation status of Wnt antagonist genes changed between GA and GAC, and (2) the relationship between the pathological characteristics of gastric neoplasia, β-catenin mutational status, and expression and methylation status of Wnt antagonist genes.

Methods

Specimens

This study was approved by the local ethics committee. Twenty-four samples of primary GACs and corresponding normal gastric tissues (NGTs) were obtained by radical or partial gastrectomy. Samples of 24 low-grade adenomas (LGAs) and 24 high-grade adenomas (HGAs) were obtained from 48 patients undergoing gastroscopic polypectomy. Corresponding periadenomatous NGTs were obtained by endoscopic gastric biopsies. Histological analysis of selected biopsy materials showed that these samples contained 40–80% of epithelial tissues. The cases were grouped according to the Vienna Classification [22]. In the periadenomatous NGTs, grade of inflammation, glandular atrophy, and intestinal metaplasia were classified according to the updated Sydney System [23]. Half of each specimen was fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax. Sections (5 μm) were stained with hematoxylin and eosin for histological evaluation. Snap-frozen samples were stored at −80°C until analysis.

Immunohistochemistry

Five-micrometer-thick sections from formalin-fixed, paraffin-embedded (FFPE) tissues were used for immunohistochemical staining with anti-β-catenin (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated for 2 h at room temperature. The avidin¬–biotin–peroxidase complex procedure (ABC standard; Vector Laboratories, Burlingame, CA, USA) was performed. Peroxidase activity was detected with 3,3'-diaminobenzidine tetrachloride as the substrate.

β-catenin was expressed in the cytoplasm and cell membrane. The immunoreactive score (IRS) of β-catenin was compiled semiquantitatively. Staining intensity of β-catenin was classified as: 0, no staining; 1, weak; 2, moderate; and 3, strong. The area of positivity was assessed by providing values of 0 (focal or <10%), 1 (10–30%), 2 (30–50%) and 3 (>50%). The IRS was calculated by adding staining intensity and the percentage of positivity, which could range from 0 to 6. It was classified as "weak" pattern if IRS was <3; otherwise, it was classified as "strong" pattern. When the immunohistochemical test showed both strong membrane staining and weak cytoplasmic staining, this was classified as the "normal" pattern, and samples with other staining patterns were classified as a "disordered" pattern. Immunostaining of β-catenin was assessed by 2 independent observers blinded to patient clinical outcome and local staging.

DNA and RNA isolation

DNA and RNA from cell lines were isolated using TRIzol Reagent (Life Technologies, Breda, The Netherlands) [24]. DNA from FFPE material was isolated after macro-dissection as de-

scribed previously [25].

Analysis of the β-catenin gene

For β-catenin, a genomic polymerase chain reaction (PCR) fragment including exon 3, which was previously found to contain activating mutations, was amplified as described previously [26]. The PCRs were performed in the presence of high-fidelity Primestar DNA polymerase (HotStart version; TaKaRa, Dalian, China). Direct sequencing was performed using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). PCR and sequence analysis of mutated samples were repeated twice to exclude PCR errors.

Reverse transciption-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 μg of total RNA. The PCR products were analyzed on a 2% agarose gel. qRT-PCR was performed using iCycler with the iQ SYBR Green Supermix (Bio-Rad) and the same gene-specific primers; β-actin was used as an internal control [27]. The relative quantity of the transcripts was calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔCt was determined by subtracting the average β-actin Ct value from the average target Ct value.

Methylation-specific PCR (MSP)

Genomic DNA was modified with sodium bisulfite using a commercial kit (Invitrogen, Carlsbad, CA, USA). The targeted genes used in this study were APC, sFRP-1, DKK-1 and WIF-1 The first universal primer set covered no CpG sites in either the forward or reverse primer and amplified a DNA fragment of the promoter region containing several sites. A second round of nested MSP or unmethylation-specific PCR (USP) was done using the universal PCR products as templates. Primer sequences designed for MSP and USP of the Wnt antagonist genes have been reported previously [18]. For semiquantitative MSP analysis, a preliminary suitable number of PCR cycles for each primer set was carried out to determine the linear range of the reaction. The PCR products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide, and DNA bands were visualized by UV light. In samples with a positive MSP band, the relative methylation ratio was determined after the MSP or USP product was electrophoresed in nondenaturing 12% polyacrylamide gels. The area under the curve (AUC) corresponding to each band was calculated using ImageJ software [4] and the relative methylation level was determined [MSP ratio = MSP band density/ (MSP band density+USP band density)] as reported previously [28,29].

Statistical analysis

Descriptive statistics including mean and standard deviation (SD) were calculated, and bar graphs were generated to summarize the results. The differences in expression levels were analyzed using the t-test and Welch's method (2-sided). The categorical variables were analyzed using the $\chi 2$ or Fisher's exact test. Multivariate analysis for dependent categorical variables was performed by binomial logistic regression analysis. $P < 0.05$ was considered statistically significant. All data were analyzed with SPSS for Windows version 13.0 (SPSS, Chicago, IL, USA).

Results

Clinical and histological characteristics

The clinical and histological characteristics of the patients are shown in Table 1. There were n men and n women, with a mean age of 56 years (range: n–n years). Adenomas were more prevalently polypoid than flat. The mean adenoma size was 9.4 mm (range: 6–25 mm). The majority of cases were <10 mm. There were various grades of inflammation in all periadenomatous NGTs, but there were 5 cases of NGTs accompanying intestinal metaplasia and 3 cases accompanying glandular atrophy. The grade of inflammation was significantly related to size (P = 0.000) and grade (P = 0.006) of GAs.

Variables	Total cases (n=48)	Grade of adenomas	
	n (%)	Low (n=24)	High(n=24)
		n (%)	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)
NGT, corresponding normal gastric tissues			

Table 1: Clinicopathological characteristics of the 48 gastric adenomas

Immunohistochemistry for β-catenin expression in different gastric epithelial tissues

To examine Wnt/β-catenin signaling status in different gastric epithelial tissues, we first assessed β-catenin localization in clinical tissues. In most NGTs, β-catenin was expressed strongly at the cell membrane and was also observed faintly in the cytoplasm, as reported for the colorectal epithelium [30]. For LGAs, in 14 cases (58.3%), β-catenin was strongly expressed at the cell membrane, and was classified as a normal pattern, while the remaining 10 cases (41.7%) were classified as a disordered pattern. These 10 cases all exhibited strong β-catenin expression in the cytoplasm. Weak expression of β-catenin was observed in the cell membrane and diffuse strong cytoplasmic staining was apparent in 20 (83.3%) HGAs, sometimes with weak nuclear staining. β-Catenin expression seldom was observed in the cell membrane and strong cytoplasmic or nuclear staining was apparent in 22 (91.7%) cases of gastric cancer (Fig. 1). The frequency of disordered expression of β-catenin was significantly greater in GACs compared to NGTs ($P < 0.005$) (Table 2). However, there was no difference between HGAs and GACs.

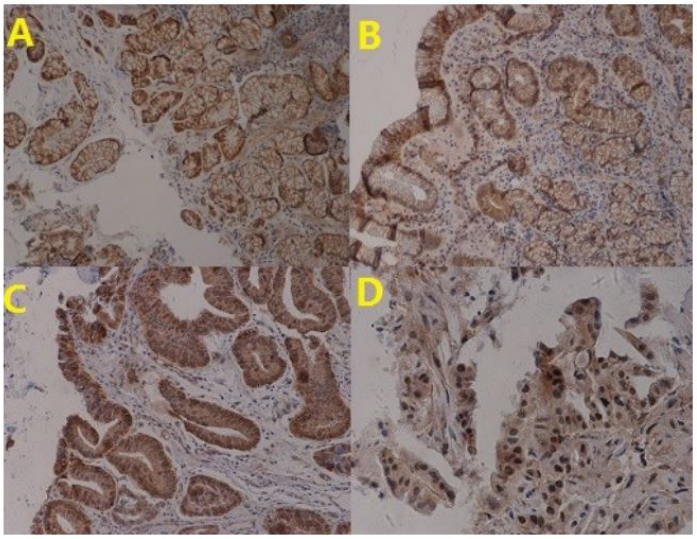


Figure 1. β-Catenin expression in NGTs, GAs and GCs. (A) NGTs immunostained for β-catenin. Note the presence of strong membrane staining and weak cytoplasmic staining. Hematoxylin counterstain; original magnification: ×200. (B) LGAs immunostained for β-catenin. Note the presence of moderate membrane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

Type of tissues	Cases	Normal	Abnormal
NGTs	72	69（95.8）	3（4.2）
Gastric adenoma	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, $P = 0.002$. LGAs versus NGTs, $P < 0.001$. HGAs versus NGTs, $P < 0.001$. GACs versus NGTs, $P < 0.001$. GACs versus LGAs, $P < 0.001$. GACs versus HGAs, $P = 0.394$. NGT corresponding normal gastric tissues, LGA low-grade adenoma, HGA high-grade adenoma, GAC primary gastric adenocarcinoma			

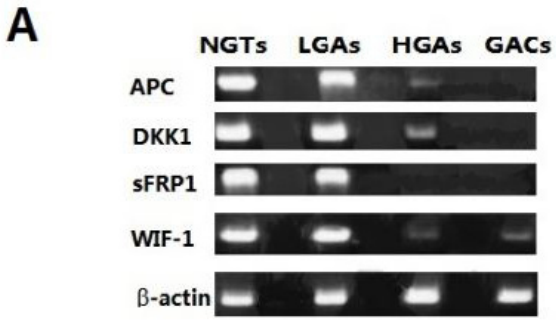
Table 2: β-catenin expression in different gastric epithelial tissues, n(%)

Mutation in exon 3 of β-catenin

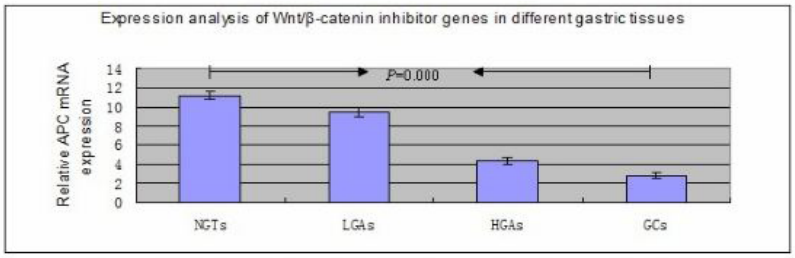
No mutations in exon 3 of β-catenin were found in LGAs, HGAs or NGTs. Only one gene mutation was detected in 24 GACs (4.2%). Sequencing analysis revealed a gene mutation with an in-frame 6 bp deletion (delGGTGCC, Gly38Ala39) at codons 38 and 39.

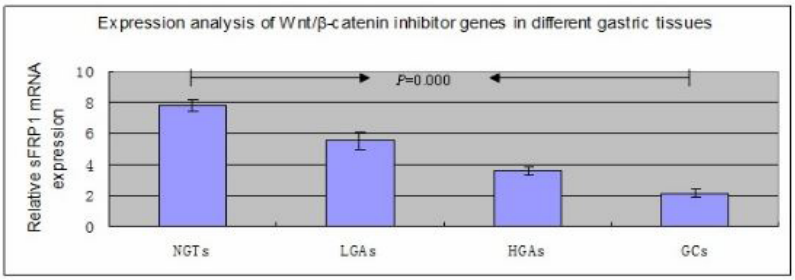
mRNA expression of Wnt antagonist genes

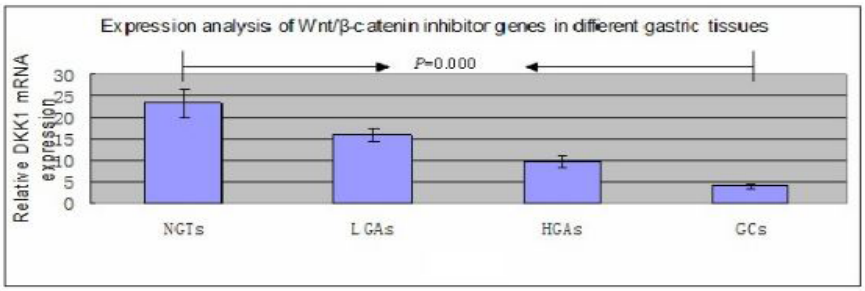
To determine whether the silencing of negative regulators of the pathway contributed to the aberrant activation of Wnt/β-catenin signaling, we examined the expression of representative Wnt/β-catenin signaling antagonists, APC, DKK-1, sFRP-1 and WIF-1 using RT–PCR. Expression of Wnt antagonist genes was weakened in HGAs and GACs (Fig. 2A). To confirm these results, mRNA expression levels of the above-mentioned genes were also investigated by qRT-PCR. mRNA expression was significantly lower in GACs than LGAs ($P = 0.000$) (Fig. 2B).


A
NGTs
LGAs
HGAs
GACs
APC
DKK1
sFRP1
WIF-1
β-actin

B


Expression analysis of Wnt/β-catenin inhibitor genes in different gastric tissues
Relative APC mRNA expression
P=0.000
NGTs
LGAs
HGAs
GCs


Expression analysis of Wnt/β-catenin inhibitor genes in different gastric tissues
Relative sFRP1 mRNA expression
P=0.000
NGTs
LGAs
HGAs
GCs


Expression analysis of Wnt/β-catenin inhibitor genes in different gastric tissues
Relative DKK1 mRNA expression
P=0.000
NGTs
LGAs
HGAs
GCs

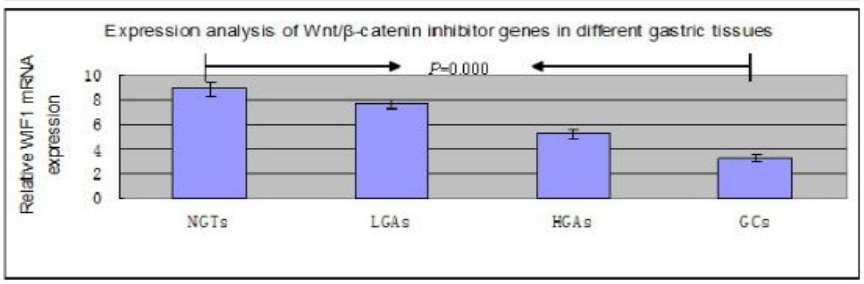

Expression analysis of Wnt/β-catenin inhibitor genes in different gastric tissues
Relative WIF1 mRNA expression
P=0.000
NGTs
LGAs
HGAs
GCs

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 significant decreased from LGAs to GCs (P = 0.000). The relative amount of mRNA expression was calculated by the comparative ΔΔCt 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.

MSP for Wnt antagonist genes

To determine whether the reduction of expression of the aforementioned Wnt/β-catenin signaling pathway antagonists was due to CpG island methylation, we examined the methylation status of the 5' regions of these genes using MSP analysis and bisulfite sequencing in four kinds of gastric tissues. Compare to NGTs and LGAs, the APC, DKK-1, sFRP-1and WIF-1 promoter methylation levels were significantly elevated in GACs and HGAs (Table 3). There was only hypermethylation of APC in NGTs (6/72, 8.3%). For APC promoter methylation levels, there was no significant difference between NGTs and LGAs (P = 0.252). For APC, DKK-1, sFRP-1 and WIF-1 promoter methylation levels, there were no significant differences between HGAs and GACs (PAPC = 1.0, PDKK1 = 0.568, PsFRP1 = 0.561 and PWIF1 = 0.251). Promoter methylation of the 4 genes correlated with abnormal expression of β-catenin (Table 4). The number of concurrently methylated genes increased from NGTs to GACs. GACs and HGAs had more concurrently methylated genes than NGTs and LGAs (P = 0.000) (Fig. 3). There was no significant difference in the number of concurrently methylated genes between GACs and HGAs (P = 0.284) or between NGTs and LGAs (P = 0.162). Thus, there was a significant difference in the number of concurrently methylated genes between LGAs and HGAs (P = 0.000). The levels of methylation of the above genes correlated with the degree of local inflammation (P = 0.000, r = 0.287).

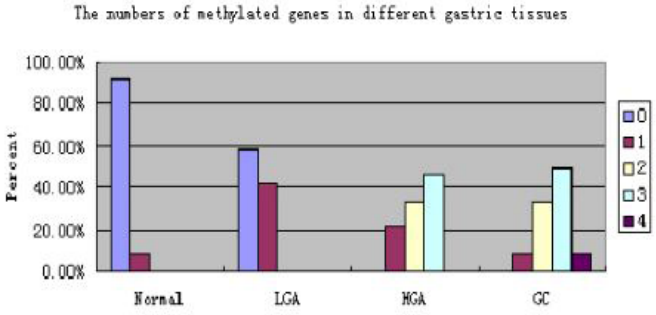


Figure 3. Summary of the methylation profile in NGTs, LGAs, HGAs and GACs. Concurrently methylated genes increased from NGTs to GACs. GACs and HGAs had more concurrently methylated genes than NGTs and LGAs (P = 0.000). There was no significant difference in the number of concurrently methylated genes between GACs and HGAs (P = 0.284) or between NGTs and LGAs (P = 0.162).GAC = gastric adenocarcinoma; HGA = high-grade adenoma; LGA = low-grade adenoma; NGT = normal gastric tissue.

		NGTs	(%)		LGAs	HGAs	GACs
	LGAs	HGAs	GCs	Total			
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	o	0	2/24(8.3)	12/24(50.0)	15/24(62.5)
NGTs:LGAs, PAPC=0.252, PDKK1= PsFRP1= PWIF1=0.013 HGAs:GACs, PAPC=1.0, PDKK1=0.568, PsFRP1=0.561, PWIF1=0.251 NGTs:HGAs and NGTs:GACs, PAPC= PDKK1= PsFRP1= PWIF1= 0.000 LGAs:GACs, PAPC= PsFRP1= PWIF1= 0.000, PDKK1=0.003 LGAs:HGAs, PAPC= PsFRP1= 0.000, PDKK1=0.016, PWIF1=0.001 NGT:corresponding normal gastric tissues, LGA: low-grade adenoma, HGA: high-grade adenoma, GAC: primary gastric adenocarcinoma							

Table 3: Frequency of promoter methylation of the Wnt antagonist genes 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						

Table 4: Correlation between status of the Wnt antagonist genes methylation and immunohistochemical expression of β-catenin in different gastric epithelial tissues

Discussion

β-Catenin is a key mediator of the canonical Wnt signaling pathway [31]. The accumulation of β-catenin in the cytoplasm and nucleus is a required primary step for activation of the pathway and hyperexpression of the target gene, which is induced by the β-catenin/TCF/LEF transcriptional complex [32]. In our study, ectopic expression of β-catenin in NGTs, LGAs, HGAs and GACs was detected in 4.2%, 41.7%, 83.8% and 91.7%, respectively. The difference among 3 gastric tissues was significant ($P < 0.001$). However, there was no significant difference between HGAs and GACs ($P = 0.682$). The data suggest that aberrant Wnt/β-catenin expression played an important role in GAs and GACs, and the histological character of HGAs is more similar to GACs than LGAs.

In recent investigations, several antagonists of the Wnt pathway have been identified. As Wnt antagonist genes, APC, sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1 and Dkk-3 inhibit Wnt signaling by binding to Wnt molecules or the low-density lipoprotein receptor-related protein (LRP)5/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 β-catenin. Up to now, downregulation of Wnt antagonist genes has been identified in a variety of malignancies, including bladder, [18,33,34] lung [35,36] and breast [37] cancer, chronic lymphocytic leukemia, [38] and even gastric [19,20] and esophageal [39] carcinoma. In our study, mRNA expression levels of the examined Wnt-antagonist genes were significant lower in GACs than LGAs ($P = 0.000$). We analyzed the relationship between Wnt antagonist gene methylation status and ectopic expression of β-catenin in 4 gastric tissues. Compared to NGTs and LGAs, APC, DKK-1, sFRP-1and WIF-1 promoter

methylation levels were significantly elevated in GACs and HGAs ($P < 0.05$). In addition, for APC, DKK-1, sFRP-1and WIF-1 promoter methylation levels, there were no significant differences between HGAs and GACs (PAPC = 1.0, PDKK1 = 0.568, PsFRP1 = 0.561 and PWIF1 = 0.251). There was a marked concordance between promoter methylation of Wnt antagonist genes and ectopic expression of β-catenin, which indicated that hypermethylation of Wnt antagonist genes is one of the critical mechanisms for a shift of β-catenin protein from the cell membrane to the nucleus. This may be mediated through the aberrant canonical Wnt/β-catenin signal activation involved in the pathogenesis of GAs and GACs. The number of concurrently methylated genes increased from NGTs to GACs, and GACs and HGAs had more concurrently methylated genes than NGTs and LGAs ($P = 0.000$). This finding may reflect that aberrant methylation of Wnt antagonist genes was already present in the precancerous stage or at early onset of gastric cancer, and was involved in the early initiation as well as the transformation process.

In addition, Guo et al. [40] found that most of the Wnt antagonist genes that were methylated were tumor specific. However, there were some cases in which the change in methylation was present in tumor tissues as well as paired non-cancerous tissues. Given the high sensitivity of MSP analysis, it was possible that normal-appearing specimens contained few cancer cells that were undetectable by histomorphology, and hypermethylation in corresponding non-cancerous tissues may represent the appearance of premalignant lesions [40]. In the present study, hypermethylation of APC gene was discovered in NGTs, which differed from the other 3 Wnt antagonist genes. There was no significant difference between NGTs and LGAs ($P = 0.252$). In the study of Klump et al., [41] hypermethylation of the tumor suppressor gene p16, which indicates neoplastic progression in Barrett's esophagus, was detected in pathologically normal specimens from a patient who later developed dysplasia. Therefore, epigenetic inactivation of APC genes may be an aberrant and early feature of tumorigenesis in GACs.

To the best of our knowledge, no study about promoter methylation and mRNA expression of Wnt antagonist genes in GA has been reported, however, there were some studies about the correlation of promoter hypermethylation of Wnt antagonist genes with gastric cancer and esophageal carcinoma [19,20,39]. Yoshida and Saito [42] showed that 30% of GAs examined under endoscopy were associated with cancerous changes, suggesting a similar underlying pathogenesis for adenoma and carcinoma. Another study of GAs revealed a GC risk of 2.5–50% [43,44]. So far, GA is considered to be a more specific premalignant lesion than atrophic gastritis alone.[3] Therefore, we evaluated the relation between the promoter hypermethylation of 4 Wnt antagonist genes, expression and mutation of β-catenin, and histological characteristics in GAs. The abnormal expression of β-catenin in the NGTs, LGAs and HGAs was detected in 4.2%, 41.7% and 83.3%, respectively ($P < 0.001$). No mutations in exon 3 of β-catenin were found in LGAs and HGAs. Compared to LGAs, the APC, DKK-1, sFRP-1and WIF-1 promoter methylation levels were significantly elevated in HGAs ($P < 0.05$). There was a significant difference in the number of concurrently methylated genes between LGAs and HGAs ($P = 0.000$). The levels of methylation of the above genes correlated with the degree of local inflammation ($P = 0.000$, $r = 1.287$). The results showed the gastric inflammation may be a stimulant for the incident and development of gastric adenoma. The phenomena also hinted that GA was a particularly premalignant lesion that was distinct from glandular atrophy and intestinal metaplasia.

This study was limited by its small number of patients and cancer-related genes. Thus, larger multi-gene studies are needed to validate our results. 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 adenocarcinoma. In conclusion, our data suggest that hypermethylation of multiple Wnt antagonist genes plays a role in gastric tumorigenesis, contributing to irregular activation of Wnt signaling. Therefore, the hypermethylation status of Wnt antagonist genes could be an attractive target in the management of gastric neoplasia at major risk of progression to cancer and may promote objective criteria for intervention, such as endoscopic mucosal resection. In this regard, detection of hypermethylation may be developed into a diagnostic tool to identify patients at risk for further histological progression of GAC.

References

1. Correa P (1988) A human model of gastric carcinogenesis. Cancer Res 48: 3554-3560.
2. Tsujitani S, Furusawa M, Hayashi I (1992) Morphological factors aid in therapeutic decisions concerning gastric adenomas. Hepatogastroenterology 39: 56–58.
3. Abraham SC, Montgomery EA, Singh VK, Yardley JH, Wu TT (2002) Gastric adenomas: intestinal-type and gastric-type adenomas differ in the risk of adenocarcinoma and presence of background mucosal pathology. Am J Surg Pathol 26: 1276–1285.
4. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61: 759-767.
5. Anastas JN, Moon RT (2013) WNT signaling pathways as therapeutic targets in cancer. Nat Rev Cancer 13: 11–26.
6. Behrens J, vonKries JP, KuhlM, Bruhn L,WedlichD, et al. (1996) Functional interaction of ß-catenin with the transcription factor LEF-1. Nature 382: 638–642.
7. Leushacke M, Barker N (2012) Lgr5 and Lgr6 as markers to study adult stem cell roles in self-renewal and cancer. Oncogene 31: 3009-3022.
8. Ramachandran I, Thavathiru E, Ramalingam S, Natarajan G, Mills WK, et al. (2012) Wnt inhibitory factor 1 induces apoptosis and inhibits cervical cancer growth, invasion and angiogenesis in vivo.Oncogene 31:2725-2737.

9. Schepeler T, Holm A, Halvey P, Nordentoft I, Lamy P (2012) Attenuation of the beta-catenin/TCF4 complex in colorectal cancer cells induces several growth-suppressive microRNAs that target cancer promoting genes. Oncogene 31: 2750-2760.

10. Zhen-Kai Wang, Jiong Liu, Chan Liu, Fang-Yu Wang, Chun-Yan Chen, et al. (2012) Hypermethylation of adenomatous polyposis coli gene promoter is associated with novel Wnt signaling pathway in gastric adenomas . Journal of Gastroenterology and Hepatology 27: 1629–1634.

11. Yoda Y, Takeshima H, Niwa T, Kim JG, Ando T, et al. (2015) Integrated analysis of cancer-related pathways affected by genetic and epigenetic alterations in gastric cancer. Gastric Cancer 18: 65-76.

12. Loh M, Liem N, Vaithilingam A, Lim PL, Sapari NS, et al. (2014) DNA methylation subgroups and the CpG island methylator phenotype in gastric cancer: a comprehensive profiling approach. BMC Gastroenterol 14: 55-66.

13. Ying Y, Tao Q (2009) Epigenetic disruption of the WNT/beta-catenin signaling pathway in human cancers. Epigenetics 4: 307-312.

14. Esteller M, Sparks A, Toyota M, Sanchez-Cespedes M, Capella G, et al. (2000) Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res 60: 4366-4371.

15. Koch A, Weber N, Waha A, Hartmann W, Denkhaus D, et al. (2004) Mutations and elevated transcriptional activity of conductin (AXIN2) in hepatoblastomas. J Pathol 204: 546-554.

16. Tseng RC, Lin RK, Wen CK, Tseng C, Hsu HS, et al. (2008) Epigenetic silencing of AXIN2/beta TrCP and deregulation of p53-mediated control lead to wild-type beta-catenin nuclear accumulation in lung tumorigenesis. Oncogene 27: 4488-4496.

17. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, et al (2004) Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet 36: 417-422.

18. Urakami S, Shiina H, Enokida H, Kawakami T, Kawamoto K, Hirata H, et al. (2006) Combination analysis of hypermethylated Wnt-antagonist family genes as a novel epigenetic biomarker panel for bladder cancer detection. Clin Cancer Res ;12: 2109-2116.

19. Sato H, Suzuki H, Toyota M, Nojima M, Maruyama R, et al. (2007) Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. Carcinogenesis 28: 2459-2466.

20. Nojima M, Suzuki H, Toyota M, Watanabe Y, Maruyama R, et al. (2007) Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. Oncogene 26: 4699-4713.

21. Licchesi JD, Van Neste L, Tiwari VK, Cope L, Lin X, et al. (2010) Transcriptional regulation of Wnt inhibitory factor-1 by Miz-1/c-Myc. Oncogene 29: 5923-5934.

22. Schlemper RJ, Riddell RH, Kato Y, Borchard F, Cooper HS, et al. (2000) The Vienna classification of Gastrointestinal epithelial neoplasia.Gut 47: 251–255.

23. Dixon MF, Genta RM, Yardley JH, Correa P (1996) Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 20: 1161-1181.

24. Wilting SM, Snijders PJ, Meijer GA, Ylstra B, van den Ijssel PR, et al. (2006) Increased gene copy numbers at chromosome 20q are frequent in both squamous cell carcinomas and adenocarcinomas of the cervix. J Pathol 209: 220-230.

25. Voorham QJ, Carvalho B, Spiertz AJ, van Grieken NC, Mongera S, et al. (2012) Chromosome 5q loss in colorectal flat adenomas. Clin Cancer Res 18: 4560-4569.

26. Iwao K, Nakamori S, Kameyama M, Imaoka S, Kinoshita M, et al. (1998) Activation of the beta-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. Cancer Res 58: 1021–1026.

27. Deng G, Chen A, Hong J, Chae HS, Kim YS (1999) Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. Cancer Res 59: 2029-2033.

28. Enokida H, Shiina H, Igawa M, Ogishima T, Kawakami T, et al. (2004) (CpG hypermethylation of MDR1 gene contributes to the pathogenesis and progression of human prostate cancer. Cancer Res 64: 5956-5962.

29. Shiina H, Breault JE, Basset WW, Enokida H, Urakami S, et al. (2005) Functional loss of the ß-catenin gene through epigenetic and genetic pathways in human prostate cancer. Cancer Res 65: 2130-2138.

30. Sasako M, Mann GB, van de Velde CJ, Hirohashi S, Yoshida S (1998) Report of the Eleventh International Symposium of the Foundation for Promotion of Cancer Research: Basic and Clinical Research in Gastric Cancer.Jpn J. Clin. Oncol 28: 443–449.

31. Nelson WJ, Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303: 1483-1487.

32. Wong SC, Lo ES, Lee KC, Chan JK, Hsiao WL (2004) Prognostic and diagnostic significance of ß-catenin nuclear immunostaining in colorectal cancer. Clin Cancer Res 10: 1401–1408.

33. U rakami S, Shiina H, Enokida H, Kawakami T, Tokizane T, et al. (2006) Epigenetic inactivation of Wnt inhibitory factor-1 plays an important role in bladder cancer through aberrant canonical Wnt/beta-catenin signaling pathway. Clin Cancer Res 12: 383–391.

34. Marsit CJ, Karagas MR, Andrew A, Liu M, Danaee H, et al. (2005) Epigenetic inactivation of SFRP genes and TP53 alteration act jointly as markers of invasive bladder cancer. Cancer Res 65: 7081-7085.

35. Zhang YW, Miao YF, Yi J, Geng J, Wang R, et al. (2010) Transcriptional inactivation of secreted frizzled-related protein 1 by promoter hypermethylation as a potential biomarker for non-small cell lung cancer. Neoplasma 57: 228-233.

36. Yang TM, Leu SW, Li JM, Hung MS, Lin CH, et al. (2009) WIF-1 promoter region hypermethylation as an adjuvant diagnostic marker for non-small cell lung cancer-related malignant pleural effusions. J Cancer Res Clin Oncol 135: 919-924.

37. Veeck J, Wild PJ, Fuchs T, Schüffler PJ, Hartmann A, et al. (2009) Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (Dkk3) promoter methylation in human breast cancer. BMC Cancer 9:217-230.

38. Chim CS, Pang R, Liang R (2008) Epigenetic dysregulation of the Wnt signalling pathway in chronic lymphocytic leukaemia. J Clin Pathol 61: 1214–1219.

39. Chan SL, Cui Y, van Hasselt A, Li H, Srivastava G, et al. (2007) The tumor suppressor Wnt inhibitory factor 1 is frequently methylated in nasopharyngeal and esophageal carcinomas.Lab Invest 87: 644–650.

40. Guo W, Dong Z, He M, Guo Y, Guo J, et al. (2010) Aberrant methylation of thrombospondin-1 and its association with reduced expression in gastric cardia adenocarcinoma. J Biomed Biotechnol 2010: 721485.

41. Klump B, Hsieh CJ, Holzmann K, Gregor M, Porschen R (1998) Hypermethylation of the CDKN2/p16 promoter during neoplastic progression in Barrett's esophagus. Gastroenterology 115: 1381–1386.

42. Yoshida S, Saito D (1996) Gastric premalignancy and cancer screening in high-risk patients. Am J Gastroenterol 91: 839-843.

43. Pisano R, Llorens P, Backhouse C, Palma M (1996) [Anatomopathological study of 86 gastric adenomas. Experience in 14 years]. Rev Med Chil 124: 204-208.

44. Stolte M (1995) Clinical consequences of the endoscopic diagnosis of gastric polyps. Endoscopy 27: 32-37.