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RUNX3 methylation and expression associated with advanced precancerous gastric lesions in a Chinese population

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Runt-related transcription factor 3 (RUNX3) is a tumor suppressor of gastric cancer. Our study aimed to investigate the correlation of *RUNX3* methylation, expression and the risk of advanced gastric lesions, based on a high-risk population in Linqu County, Shandong Province, China. Methylation status of *RUNX3* was determined by methylation-specific polymerase chain reaction, and expression was detected by immunohistochemical analysis in 1113 subjects with different gastric lesions. Results showed that the frequency of *RUNX3* methylation was significantly increased in subjects with advanced gastric lesions. The odds ratios (ORs) were 2.09 [95% confidence interval (CI): 1.49–2.94] for intestinal metaplasia (IM), 3.22 (95% CI: 2.33–4.47) for indefinite dysplasia (Ind DYS) and 2.03 (95% CI: 1.23–3.37) for dysplasia (DYS) compared with superficial gastritis/chronic atrophic gastritis. Stratified analysis indicated that the frequency of *RUNX3* methylation was higher in subjects with *Helicobacter pylori* infection (OR, 2.74; 95% CI: 2.00–3.76). Moreover, there was a reverse grade-response relationship between the level of RUNX3 expression and risk of gastric lesions. Among subjects with mild, moderate or heavy expression, the risk was decreased by 41, 59 or 80% for IM ($P_{trend} < 0.0001$); 40, 64 or 74% for Ind DYS ($P_{trend} < 0.0001$) and 28, 59 or 51% for DYS ($P_{trend} = 0.045$), respectively. Furthermore, RUNX3 expression was negatively associated with increased frequency of *RUNX3* methylation (OR, 0.76; 95% CI: 0.59–0.98). These findings suggest that RUNX3 may play important roles in the development of advanced gastric lesions.

Introduction

Gastric cancer (GC) is among the leading causes of cancer death worldwide and its etiologic factors remain to be elucidated further (1). Our 4.5 year gastroscopy-based cohort study in Linqu County, Shandong Province, China, an area with one of the highest mortality rates of GC in the world, revealed that the risk of GC was markedly increased by baseline histopathologic severity and the odds ratios (ORs) were 29 for subjects with mild dysplasia (DYS) or deep intestinal metaplasia (IM) and 104 for moderate or severe DYS (2,3), suggesting a multistep process of gastric carcinogenesis (4). However, the mechanisms regarding transformation of gastric mucosa from early to advanced gastric lesions or GC are still unclear. Therefore, investigation of the genetic and epigenetic alterations involved in the progression of precancerous gastric lesions would enhance the understanding of the carcinogenesis process of GC.

Abbreviations: CAG, chronic atrophic gastritis; CI, confidence interval; DYS, dysplasia; GC, gastric cancer; IM, intestinal metaplasia; Ind DYS, indefinite dysplasia; OR, odds ratio; PCR, polymerase chain reaction; RUNX3, runt-related transcription factor 3; SG, superficial gastritis;; TGF-β, transforming growth factor-beta.

Runt-related transcription factor 3 (*RUNX3*) is a tumor suppressor of GC and an important target of transforming growth factor-beta (TGF-β) superfamily signaling (5–8). Studies have shown that RUNX3 can regulate cell proliferation and apoptosis in gastric epithelial cells, and gastric epithelia of *Runx3*$^{-/-}$ animals become insensitive to the tumor suppressor activity of TGF-β pathway and exhibit hyperplasia (5–7,9–11). Moreover, RUNX3 could modulate differentiation of gastric epithelial cells. In *Runx3*$^{-/-}$ animals, some gastric epithelial cells can transdifferentiate into intestinal type cells, suggesting that RUNX3 may play an important role in the gastric carcinogenesis (12,13). It has also been proven that loss of RUNX3 expression due to abnormal methylation of the CpG island around the P2-promoter and loss of heterozygosity may be involved in the development of GC (6,14–16). Thus, loss of RUNX3 expression may be an important step in gastric carcinogenesis.

Although several hospital-based studies have focused on the relationship of *RUNX3* methylation or expression with some categories of gastric lesions, further population-based study is required to testify the role of RUNX3 in the transition of precancerous gastric lesions (17–20). Moreover, because our previous studies showed that *Helicobacter pylori* could induce p*16* and *COX-2* methylation (21,22), we were also interested in assessing whether *H.pylori* could induce the *RUNX3* methylation and modify the relationship between methylation and risk of advanced gastric lesions.

In the present study, we investigated *RUNX3* methylation and expression in a spectrum of gastric lesions, including superficial gastritis (SG), chronic atrophic gastritis (CAG), IM, indefinite dysplasia (Ind DYS) and DYS, and evaluated the association of abnormal methylation or protein expression with precancerous gastric lesions, as well as *RUNX3* methylation with *H.pylori* infection in a high-risk population in Linqu County.

Materials and methods

Study population

In 2002, we launched a baseline study of endoscopy for an intervention trial at 12 villages selected at random in Linqu County, Shandong Province, China. We identified 3167 subjects aged 35–64 years, who met the inclusion criteria. Of them, 2734 were eligible for the endoscope examination and 2638 subjects volunteered to participate in the endoscope examination representing 83.2% of the eligible residents. Each participant was interviewed by trained investigators using a questionnaire to obtain information on cigarette smoking and alcohol consumption during the endoscope examination.

For the present study, 1113 subjects with a spectrum of precancerous gastric lesions were selected. The study protocol was approved by the Institutional Review Board of Peking University Cancer Hospital, and all subjects gave written informed consent.

Histopathology

Endoscopy procedures have been described in detail elsewhere (3). The biopsy specimens were taken at five standard sites according to the Updated Sydney System (23), including two from the antrum (one from the greater curvature and one from smaller curvature), one from the angulus and two from the body (one from the greater curvature and one from smaller curvature). Tissues were formalin fixed, paraffin embedded, then sliced and dyed by hematoxylin & eosin. Each slide was reviewed by a panel of three senior pathologists based on the Updated Sydney System and Padova International Classification (23,24) and diagnosed as SG, CAG, IM, Ind DYS or DYS. Each biopsy was given a global diagnosis based on the most severe lesion in the biopsy. Each participant was assigned a global diagnosis based on the most severe diagnosis among any of the five biopsies. The biopsy with most severe diagnosis was selected for each subject to evaluate the *RUNX3* methylation and expression status.

^{13}C-urea breath test

Helicobacter pylori infection was determined by ^{13}C-urea breath test as described previously (25). Briefly, baseline breath samples were collected for

each participant after an overnight fast. Then, participants were required to take 80 mg ^{13}C-urea (^{13}C abundance > 99%, purity > 98.5%), and the second breath samples were collected after 30 min. The content of $^{13}CO_2$ in the two vials of breath samples for each participant was analyzed by a gas isotopic ratio mass spectrometer. The subject was considered *H.pylori* positive if the concentration of $^{13}CO_2$ increased >4 parts per 1000 when comparing the second breath sample with the baseline one.

DNA extraction and bisulfite treatment

High molecular weight genomic DNA was isolated and treated with bisulfite as previously reported (26). After being deparaffinized by xylene and rehydrated with graded ethanol, tissue sections were digested by lysis buffer containing proteinase K at 50°C overnight and then modified with sodium bisulfite to convert the unmethylated cytosines to uridines. Bisulfite-modified DNA was then purified with a genomic DNA purification kit (Promega, Madison, WI).

Methylation-specific polymerase chain reaction

The methylation status of *RUNX3* was determined by methylation-specific polymerase chain reaction (PCR) (27). The *RUNX3*-5M (5′-ATAATAGCGGTCGTTAGGGCGTCG-3′) and -3M (5′-GCTTCTACTTTCCCGCTTCTCGCG-3′) primer set was used for detecting methylated DNA (115 bp). The *RUNX3*-5U (5′-ATAATAGTGGTTGTTAGGGTGTTG-3′) and -3U (5′-ACTTCTACTTTCCCACTTCTCACA-3′) primer set was used for detecting unmethylated DNA (115 bp) (28). Methylation-specific PCR for each sample was accomplished with a 20 μl reaction mixture in 1× reaction buffer containing 10 ng of bisulfite-modified genomic DNA, 0.25 μM of each primer, 0.2 mM of diethylnitrophyenyl thiophosphate, and 0.5 U *Taq* DNA polymerase (QIAGEN GmbH, Hilden, Germany). Methylation-specific PCR was done for amplification of the methylated *RUNX3* under the following procedures: denaturing at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 63°C for 45 s and elongation at 72°C for 1 min, with a final extension at 72°C for 10 min.

Distilled water was used as blank control. *RUNX3*-methylated human gastric adenocarcinoma cell line (AGS) with no RUNX3 expression and *RUNX3*-unmethylated GC cell line MKN45 with RUNX3 expression were applied as positive and negative control, respectively (6,18,20), of which the *RUNX3* methylation or unmethylation status has been testified by sequencing. PCR products were analyzed on 8% polyacrylamide gel electrophoresis stained by ethidium bromide and visualized under ultraviolet illumination.

Immunohistochemical analysis

We applied immunohistochemical analysis to determine the RUNX3 expression status (14). After being deparaffinized by xylene and rehydrated by graded ethanol, the tissue sections were treated with 3% hydrogen peroxide to block the endogenous peroxidase activity. Then the slides were retrieved for 1.5 min by heat-induced epitope retrieval with sodium citrate buffer (0.01 M, pH 6.0) in autoclave. The tissues were blocked with 5% degreased milk at 37°C for 2 h and then incubated with a rabbit polyclonal antibody against human RUNX3 in a 1:300 dilution (Active Motif, Carlsbad, CA) at 4°C overnight. Then, we treated the slides with biotin goat-anti-rabbit IgG and horseradish peroxidase–streptavidin (both from Zymed, San Diego, CA) in sequence, at room temperature for 15 min for each one. The slides were stained with diaminobenzidine and visualized under the microscope. The immunohistochemical expression of RUNX3 was examined independently by two investigators without the knowledge of the methylation status (supplementary figure). The percentage of positive cells was graded semiquantitatively, and each sample was assigned to one of the following categories: negative (<5% positive cells), mild (5–25% positive cells), moderate (25–50% positive cells) and heavy (>50% positive cells).

Statistical analysis

Because only 41 subjects were diagnosed as SG, therefore, SG and CAG were combined as the reference group (SG/CAG). The age of subjects was classified into two categories: <50 and ≥50 years.

With SG/CAG as the reference, we utilized unconditional logistic regression model to calculate the ORs and 95% confidence intervals (CIs) for association of *RUNX3* methylation or expression with the risk of advanced gastric lesions (IM, Ind DYS and DYS), adjusting for age, sex, *H.pylori* infection, smoking and drinking status. We also analyzed the association of *RUNX3* methylation or expression with one baseline characteristic after adjusting for others by unconditional logistic regression model, with methylation or expression as the dependent variable. Linear trend test was applied to evaluate the changing trend in risk for advanced gastric lesions with increasing RUNX3 expression by scoring the expression categories, assigning 1–4 for negative, mild, moderate or heavy expression, respectively. Then, the scores were entered into the logistic regression model as an ordinal term to calculate the *P* value for trend (P_{trend}). We carried out unconditional logistic regression to do association analyses in stratification by *H.pylori* infection and evaluate the association of *RUNX3* methylation with protein expression. All statistical analyses were carried out using Statistical Analysis System (SAS, version 9.1; SAS Institute, Cary, NC). All statistical tests were two tailed, and the significance level was set at $P < 0.05$.

Results

A total of 1113 subjects (573 males and 540 females) were enrolled in our study including 41 subjects with SG, 240 with CAG, 308 with IM, 433 with Ind DYS and 91 with DYS. Because few subjects were diagnosed with SG, they were combined with CAG as the reference (SG/CAG) in the following analysis. These 1113 participants had completed data on age, sex, *H.pylori* infection and tobacco smoking. Data on alcohol drinking were available for 1080 participants. The frequency distribution of baseline characteristics in subjects with different gastric lesions was listed in Table I.

We first evaluated the association between *RUNX3* methylation and risk of precancerous gastric lesions. The methylation frequency of *RUNX3* varied markedly by histological status, which was 39.9% in subjects with SG/CAG, 56.2% in IM, 69.3% in Ind DYS and 57.1% in DYS, respectively. Multinomial logistic regression analysis showed that the risks of advanced gastric lesions were significantly increased in subjects having methylated *RUNX3* compared with unmethylated *RUNX3*. The ORs were 2.09 (95% CI: 1.49–2.94) for IM, 3.22 (95% CI: 2.33–4.47) for Ind DYS and 2.03 (95% CI: 1.23–3.37) for DYS, respectively (Table II).

Table I. Selected characteristics of the study participants with different gastric lesions

	Total, n = 1113 [n (%)]	SG/CAG, n = 281 [n (%)]	IM, n = 308 [n (%)]	Ind DYS, n = 433 [n (%)]	DYS n = 91 [n (%)]
Age (years)					
<50	540(48.5)	129 (45.9)	151(49.0)	225 (52.0)	35(38.5)
≥50	573 (51.5)	152 (54.1)	157 (51.0)	208 (48.0)	56 (61.5)
Sex					
Male	573 (51.5)	157 (55.9)	146 (47.4)	214 (49.4)	56 (61.5)
Female	540 (48.5)	124 (44.1)	162 (52.6)	219 (50.6)	35 (38.5)
Helicobacter pylori infection					
Positive	918 (82.5)	223 (79.4)	221 (71.8)	404 (93.3)	70 (76.9)
Negative	195 (17.5)	58 (20.6)	87 (28.2)	29 (6.7)	21 (23.1)
Smoking					
Yes	396 (35.6)	104 (37.0)	92 (29.9)	151 (34.9)	49 (53.8)
No	717 (64.4)	177 (63.0)	216 (70.1)	282 (65.1)	42 (46.2)
Drinking					
Yes	401 (36.0)	105 (37.4)	100 (32.5)	154 (35.6)	42 (46.1)
No	679 (61.0)	168 (59.8)	199 (64.6)	269 (62.1)	43 (47.3)
Missing	33 (3.0)	8 (2.8)	9 (2.9)	10 (2.3)	6 (6.6)

Table II. *RUNX3* methylation status of study participants with different gastric lesions

Methylation status	SG/CAG	IM		Ind DYS		DYS	
	n (%)	*n* (%)	OR[a] (95% CI)	*n* (%)	OR[a] (95% CI)	*n* (%)	OR[a] (95% CI)
Unmethylated	169 (60.1)	135 (43.8)	1.00	133 (30.7)	1.00	39 (42.9)	1.00
Methylated	112 (39.9)	173 (56.2)	2.09 (1.49–2.94)	300 (69.3)	3.22 (2.33–4.47)	52 (57.1)	2.03 (1.23–3.37)

[a]Unconditional logistic regression, adjusted for age, sex, *Helicobacter pylori* infection, drinking and smoking status.

Table III. Comparison of *RUNX3* methylation status between subjects with different gastric lesions by *Helicobacter pylori* infection

	SG/CAG *n* (%)	IM/Ind DYS/DYS *n* (%)	OR[a] (95% CI)
H.pylori negative			
Unmethylated	36 (62.1)	64 (46.7)	1.00
Methylated	22 (37.9)	73 (53.3)	1.93 (0.94–3.94)
H.pylori positive			
Unmethylated	143 (61.4)	243 (35.0)	1.00
Methylated	90 (38.6)	452 (65.0)	2.74 (2.00–3.76)

[a]Unconditional logistic regression, adjusted for age, sex, drinking and smoking status.

We also assessed the relationships between *RUNX3* methylation and baseline characteristics and found that *RUNX3* methylation was not associated with age, sex, cigarette smoking or alcohol drinking (data not shown). However, there was a significant association between *RUNX3* methylation and *H.pylori* infection. Compared with *H.pylori*-negative subjects, the OR of *RUNX3* methylation was elevated in subjects with *H.pylori* infection (OR, 1.42; 95% CI: 1.02–1.96). Moreover, stratified analysis indicated a significantly increased risk of advanced gastric lesions (IM/Ind DYS/DYS) in subjects with methylated *RUNX3* and *H.pylori* (OR, 2.74; 95% CI: 2.00–3.76) (Table III).

We further evaluated RUNX3 expression level in different gastric lesions. The frequencies of subjects with RUNX3 mild, moderate and heavy expression were 52.8, 23.8 and 8.3%, respectively. We found a reverse grade-response association between the levels of RUNX3 expression and risk of advanced gastric lesions (Table IV). For subjects with mild, moderate or heavy expression, the ORs were decreased from 0.59 (95% CI: 0.34–1.00) and 0.41 (95% CI: 0.23–0.73) to 0.20 (95% CI: 0.09–0.44) for IM ($P_{trend} < 0.0001$); from 0.60 (95% CI: 0.36–1.01) and 0.36 (95% CI: 0.21–0.63) to 0.26 (95% CI: 0.13–0.52) for Ind DYS ($P_{trend} < 0.0001$) and from 0.72 (95% CI: 0.34–1.55) and 0.41 (95% CI: 0.17–0.99) to 0.49 (95% CI: 0.18–1.34) for DYS ($P_{trend} = 0.045$), respectively.

We were also interested to investigate whether *RUNX3* methylation was associated with protein expression. Compared with subjects having negative or mild RUNX3 expression, the frequency of *RUNX3* methylation was significantly decreased among subjects with moderate or heavy expression (OR, 0.76; 95% CI: 0.59–0.98) (Table V).

Discussion

As part of a series of studies conducted in Linqu County, a high-risk area of GC in China (2,3,29–31), we evaluated *RUNX3* methylation and expression and their association with advanced gastric lesions. We found the frequency of *RUNX3* methylation was markedly increased in subjects with advanced gastric lesions, and *H.pylori* infection was associated with *RUNX3* methylation. Furthermore, there was a significantly reverse grade-response relationship between the levels of RUNX3 expression and risk of gastric lesions. These results provide the evidence that downregulation of RUNX3 expression by promoter methylation may play important roles in the transition of precancerous gastric lesions.

Members of *RUNX* gene family play crucial roles in normal development and carcinogenesis (32), and *RUNX3* is related to neurogenesis and thymopoiesis and functions as a tumor suppressor involved in the development of GC (6,8,32–34). RUNX3 can form complex with Smads to transmit TGF-β/activin signals and act as a significant target molecule of TGF-β signaling pathway (6,7,9,10). In the *Runx3*$^{-/-}$ animals, gastric epithelia exhibited hyperplasia and epithelial cells transdifferentiated into intestinal type cells, inducing the IM of gastric mucosa (5–7,9–13). RUNX3 is extensively expressed in the cytoplasm and nucleus of normal gastric epithelial cells, and loss of RUNX3 expression may play an important role in the development of GC (5–7,14).

Accumulating evidences from basic researches suggest that the abnormal methylation of *RUNX3* could induce the downregulation of RUNX3 expression and contribute to gastric tumorigenesis (6,7,14,18,35). Several hospital-based studies have shown that the frequency of *RUNX3* methylation was significantly increased in GC or advanced gastric lesions, such as IM (17,18). However, in view of their small sample size, restricted types of gastric lesions, and generally cancer surrounding tissues, extrapolation of those results may be limited. A population-based study is required to testify the role of *RUNX3* methylation in the transition of precancerous gastric lesions. In a population-based approach, our study found an elevated frequency of *RUNX3* methylation in advanced gastric lesions and the risk of IM, Ind DYS and DYS increased significantly among subjects having methylated *RUNX3*. This result is consistent with a previous study analysing the methylation status of hospital-based chronic gastritis, IM and gastric adenoma tissues, although different primer sets were used and lower methylation frequency was obtained in that study (19).

Helicobacter pylori is considered a major risk factor for GC (1) and closely correlated with transformation of gastric lesions (29). *Helicobacter pylori* can induce the nitric oxide, which activates DNA methyltransferase, leading to the abnormal methylation of tumor suppressor genes, such as E-cadherin (36,37). Our previous studies in Linqu provided evidence that *H.pylori* could induce methylation of p*16* and *COX-2* (21,22). In the current study, our data also indicated a positive relationship between *H.pylori* infection and *RUNX3* methylation, in agreement with previous studies (38,39). Further analysis revealed that the significant correlation of *RUNX3* methylation with advanced gastric lesions existed only among subjects with *H.pylori* infection, suggesting that *H.pylori* infection could potently induce *RUNX3* methylation during the gastric carcinogenesis.

In our study, the rate of RUNX3 expression was >90% among subjects with SG/CAG and obviously reduced in advanced gastric lesions. In addition, a reverse grade-response relationship between the levels of RUNX3 expression and risk of gastric lesions was observed. It has been reported that the expression of RUNX3 is frequently suppressed in the precancerous gastric lesions such as IM (20,40). Our study provided further evidence by a large population-based study (1113 subjects) in a high-risk area of GC and strongly suggested that downregulation of RUNX3 expression may play important roles in the evolution of precancerous gastric lesions.

It remains an important question whether the downregulation of RUNX3 expression was induced by abnormal methylation of *RUNX3* in our study. Several lines of evidence indicated that *RUNX3* abnormal methylation of the CpG island or loss of heterozygosity could cause the downregulation of RUNX3 expression in GC (6,14–16). However, there are limited data on the association between RUNX3 expression

Table IV. RUNX3 expression status of study participants with different gastric lesions

RUNX3 expression	SG/CAG	IM		Ind DYS		DYS	
	n (%)	*n* (%)	OR[a] (95% CI)	*n* (%)	OR[a] (95% CI)	*n* (%)	OR[a] (95% CI)
Negative	25 (8.9)	53 (17.2)	1.00	75 (17.3)	1.00	15 (16.5)	1.00
Mild	138 (49.1)	167 (54.2)	0.59 (0.34–1.00)	234 (54.1)	0.60 (0.36–1.01)	49 (53.8)	0.72 (0.34–1.55)
Moderate	82 (29.2)	72 (23.4)	0.41 (0.23–0.73)	94 (21.7)	0.36 (0.21–0.63)	17 (18.7)	0.41 (0.17–0.99)
Heavy	36 (12.8)	16 (5.2)	0.20 (0.09–0.44)	30 (6.9)	0.26 (0.13–0.52)	10 (11.0)	0.49 (0.18–1.34)
P_{trend}			<0.0001		<0.0001		0.0455

[a]Unconditional logistic regression, adjusted for age, sex, *Helicobacter pylori* infection, drinking and smoking status.

Table V. Association of *RUNX3* methylation with expression

Characteristics	Negative or mild expression (%)	Moderate or heavy expression (%)	OR (95% CI)[a]
Unmethylated	307 (40.6)	169 (47.3)	1.00
Methylated	449 (59.4)	188 (52.7)	0.76 (0.59–0.98)

[a]Unconditional logistic regression, adjusted for age, sex, *Helicobacter pylori* infection, drinking and smoking status and histopathology.

and methylation in the precancerous gastric lesions. Although we did not determine the frequency of loss of heterozygosity, the frequency of *RUNX3* methylation was significantly decreased among subjects with moderate or heavy expression, consistent with previous studies and suggesting that *RUNX3* methylation would induce the silencing of its expression (6,14,15).

In the present study, the relatively large sample selected at random from a well-defined population allowed us to assess the role of RUNX3 in the transition of precancerous gastric lesions. However, our study still has some limitations. Because of the few incidents of GC from this population, we cannot analyse the methylation and expression status of *RUNX3* in cancer tissues. Moreover, except for methylation, loss of heterozygosity or genetic variations may also contribute to downregulation of expression in gastric lesions; therefore, we should pay attention to those effects in the future (6,41).

In conclusion, our study provided evidences on the relationship of *RUNX3* methylation or expression with precancerous gastric lesions. We found an elevated risk of advanced gastric lesions correlated with abnormal *RUNX3* methylation or decrease of expression, and *RUNX3* methylation was associated with its expression. These findings suggest that RUNX3 may play important roles in the evolution of precancerous gastric lesions. Further study on the specific mechanisms regarding the roles of RUNX3 in gastric lesions is still warranted to confirm the effect.

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