**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 in subjects with advanced gastric lesions, including superficial gastritis (SG), chronic atrophic gastritis (CAG), IM, indefinite dysplasia (Ind DYS) or 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.

**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.

**13C-urea breath test**

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

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

Wen-Qing Li, Kai-Feng Pan, Yang Zhang, Cai-Xuan Dong, Lian Zhang, Jun-Ling Ma, Tong Zhou, Ji-You Li and Wei-Cheng You

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Cancer Epidemiology and 1Department of Pathology, Peking University Cancer Hospital & Institute, 52 Fu-cheng Road, Haidian District, Beijing 100142, China

1To whom correspondence should be addressed. Tel: +86 10 88141035; Fax: +86 10 88122437; Email: weichengyou@yahoo.com

Correspondence may also be addressed to Kai-Feng Pan. Tel: +86 10 88196701; Fax: +86 10 88122437; Email: pankaifeng2002@yahoo.com

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) and 2.03 [95% CI: 1.23–3.37] 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.

**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.

**13C-urea breath test**

*Helicobacter pylori* infection was determined by 13C-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 13C-urea (13C abundance >99%, purity >98.5%), and the second breath samples were collected after 30 min. The content of 13CO2 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 13CO2 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 RUNX3-35M (5’-ATAATAACCGGT-CGTTACTCTTCCTGCCG-3’) and -3M (5’-AC-TTCTACTTTCAACTTITCACA-3’) primer set was used for detecting methylated DNA (115 bp). The RUNX3-5U (5’-ATAATGTTTGTTAGGTTGTTG-G-3’) and -3U (5’-AC- TTCTACTTTCAACTTITCACA-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 diethylthiophenyl thiophosphate, and 0.5 U Taq DNA polymerase (QIAGEN GmbH, Hilden, Germany). Methylation-specific PCR for each sample 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 unmethylated GC cell line MKN45 with RUNX3 expression were applied as positive control, and the adenocarcinoma cell line (AGS) with no RUNX3 expression and

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 by 3% hydrogen peroxide to block endogenous peroxidase activity. Then the slides were incubated with 5 min by heat-induced epitope retrieval with sodium citrate buffer (0.01 M, pH 6.0) at 95°C for 30 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 group (SG/CAG). 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: 60/5 and ≥50 years.

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

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


RUNX3 methylation/expression and precancerous gastric lesions

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

<table>
<thead>
<tr>
<th>Methylation status</th>
<th>SG/CAG (n %)</th>
<th>IM (n %)</th>
<th>OR+ (95% CI)</th>
<th>Ind DYS (n %)</th>
<th>OR+ (95% CI)</th>
<th>DYS (n %)</th>
<th>OR+ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>169 (60.1)</td>
<td>135 (43.8)</td>
<td>1.00</td>
<td>133 (30.7)</td>
<td>1.00</td>
<td>39 (42.9)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>112 (39.9)</td>
<td>173 (56.2)</td>
<td>2.09 (1.49–2.94)</td>
<td>300 (69.3)</td>
<td>3.22 (2.33–4.47)</td>
<td>52 (57.1)</td>
<td>2.03 (1.23–3.37)</td>
</tr>
</tbody>
</table>

*aUnconditional 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

<table>
<thead>
<tr>
<th>H. pylori positive</th>
<th>SG/CAG n (%)</th>
<th>IM/Ind DYS/DYS n (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>143 (61.4)</td>
<td>243 (35.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>90 (38.6)</td>
<td>452 (65.0)</td>
<td>2.74 (2.00–3.76)</td>
</tr>
</tbody>
</table>

*aUnconditional 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 infection (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 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...
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 evidence 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.

**Funding**

National High Technology R&D Program (863) grant (2006AA02A402); National Basic Research Program of China (973 Program: 2010CB529303); National Natural Science Foundation of China (30772515); A3 Foresight Program from Natural Science Foundation of China (3092114031).

**Conflict of Interest Statement:** None declared.

**References**


Received August 5, 2010; revised November 9, 2010; accepted November 19, 2010