Methylation of GATA-4 and GATA-5 and development of sporadic gastric carcinomas

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Abstract

AIM: To understand the implication of GATA-4 and GATA-5 methylation in gastric carcinogenesis.

METHODS: Methylation status of GATA-4 and GATA-5 CpG islands in human gastric mucosa samples, including normal gastric biopsies from 45 outpatients, gastric dysplasia [low-grade gastric intraepithelial neoplasia (GIN), n = 30; indefinite, n = 77], and 80 paired sporadic gastric carcinomas (SGC) as well as the adjacent non-neoplastic gastric tissues was analyzed by methylation specific polymerase chain reaction (MSP) and confirmed by denatured high performance liquid chromatography (DHPLC). Immunohistochemical staining was used to detect protein expression. The correlation between GATA-4 and GATA-5 methylation and clinicopathological characteristics of patients including Helicobacter pylori (H. pylori) infection was analyzed.

RESULTS: GATA-4 and GATA-5 methylation was frequently observed in SGCs (53.8% and 61.3%, respectively) and their corresponding normal tissues (41.3% and 46.3%) by MSP. The result of MSP was consistent with that of DHPLC. Loss of both GATA-4 and GATA-5 proteins was associated with their methylation in SGCs (P = 0.01). Moreover, a high frequency of GATA-4 and GATA-5 methylation was found in both gastric low-grade GIN (57.1% and 69.0%) and indefinite for dysplasia (42.9% and 46.7%), respectively. However, GATA-4 and GATA-5 methylation was detected only in 4/32 (12.5%) and 3/39 (7.7%) of normal gastric biopsies. GATA-4 methylation in both normal gastric mucosa and low-grade GIN was also significantly associated with H. pylori infection (P = 0.023 and 0.027, two-sides).
CONCLUSION: Epigenetic inactivation of GATA-4 (and GATA-5) by methylation of CpG islands is an early frequent event during gastric carcinogenesis and is significantly correlated with H. pylori infection.

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Key words: Dysplasia; Gastric carcinoma; GATA-4; GATA-5; Helicobacter pylori; Methylation

INTRODUCTION

GATA proteins comprise a small family of transcriptional factors defined by a highly conserved DNA-binding domain that interacts specifically with DNA cis-elements. Six distinct vertebrate GATA proteins have been characterized and classified into two subfamilies based on their structural and expression patterns. GATA-1, -2 and -3 are important in the development and differentiation of the hematopoietic cell lineage, while GATA-4, -5 and -6 guide development and differentiation in the endoderm-derived organs, and specification of proper gut embryogenesis[5,6-8]. GATA-4 deficient cells exhibit an intrinsic defect in gastric epithelial cell differentiation to parietal cells in mice[6]. Overexpression of GATA-5 induced TFF1 expression[8]. Thus, GATA-4, -5 and -6 may play a critical role in the regulation of stomach-specific gene expression and cancer development. GATA-6 may function as an oncogene since it is often upregulated in genetic information for each case was also collected according to approved institutional guidelines. The 1997 UICC-TNM criteria were used for classification of gastric cancers.

MATERIALS AND METHODS

Gastric tissue samples

Primary sporadic gastric carcinoma (SGC) and the corresponding normal gastric samples: Fresh-frozen surgical SGC and their corresponding adjacent non-neoplastic “normal” samples were from the Tissue Bank at Beijing Cancer Hospital (n = 80); 62 males and 18 females, aged 35-81 years, average age 58.5 years). The clinical and histological information for each case was also collected according to approved institutional guidelines. The 1997 UICC-TNM criteria were used for classification of gastric cancers.

Gastric dysplasia samples: Biopsies of gastric dysplasia lesions [low-grade noninvasive gastric intraepithelial neoplasia (GIN), n = 30; indefinite for dysplasia, n = 77] (51 males and 56 female, aged 41-65 years, average age 51.7 years) were collected from patients without malignant disease enrolled in a gastro-endoscopic survey in Linqu County, a rural area in Shandong Province, China which has one of the world’s highest rates of gastric cancer[13]. Histopathological diagnosis of each case was made by three senior pathologists at the Department of Pathology in Beijing Cancer Hospital, according to the Padova international classification[14]. Information on Helicobacter pylori (H. pylori) infection by the 14C-urea breath test was also collected[15]. Gastric biopsies from patients with and without chronic gastritis: Gastric mucosa biopsies were collected from outpatients undergoing gastro-endoscopic examination at Beijing Cancer Hospital. Of 45 gastric biopsies used in the present study, 18 patients were diagnosed with superficial chronic gastritis and 27 without obvious pathological changes (43 males and 2 female, aged 19-47 years, average age 30.7 years).

Informed consent was obtained from all subjects and the institutional review committee approved this study.

Detection of H. pylori

H. pylori in the normal or corresponding normal gastric samples was analyzed with a H. pylori-specific 23S rRNA-polymerase chain reaction (PCR) assay as described[16].

Methylation-specific PCR (MSP)

DNA extraction, bisulfite treatment, and MSP were performed as described previously[17,18]. The MSP primer sequences for GATA-4 and GATA-5 were as follows: GATA-4M sense, 5′-GTATAGTTCTGGAGTTTGGTGAATC-3′; GATA-4M antisense, 5′-AATTTTCTGACACTCAGTAAACCTCCG-3′; GATA-4U sense, 5′-TTTGTATAGTTTTGTTTGTATGTTGTTTATG-3′; GATA-4U antisense, 5′-TTTTGTATAGTTTTGTTTGTATGTTGTTTATG-3′; GATA-4M sense, 5′-CAACTAACAACCGAGAAACCC-3′; GATA-4U sense, 5′-TGGAGTTTTGTAGTTTTATAGTTTTGTTTATG-3′; GATA-5U antisense, 5′-CAACCAATACAACTAAACGAACGAACCG-3′; and GATA-5U sense, 5′-TTTTGTATAGTTTTGTTTGTATGTTGTTTATG-3′. Since methylation of GATA-4 and GATA-5 is a frequent event in gastric cancer cell lines[19], it is interesting to know that methylation silences of GATA-4 and GATA-5 during gastric carcinogenesis.
Sequencing of the MSP products
The MSP products for GATA-4 and GATA-5 were subcloned with the pEASY-T1 simple clone system (TransGen Biotech Company, Beijing, China), and sequenced on an ABI PRISM 3730 DNA Analyzer.

Separation and quantification of the methylated GATA-4 by denatured high performance liquid chromatography (DHPLC) [19,20]
Both methylated and unmethylated GATA-4 CpG islands were amplified by a universal primer set without CpG (GATA-4uni sense 5’-GGAGATTTTAGAGTTTGGAT-3’ and antisense 5’-CTCCCACTAACTACCTCT-3’) under thermal cycle conditions [95°C for 15 min → (95°C for 30 s → 52.8°C for 30 s → 72°C for 50 s) × 40 cycles → 72°C for 10 min]. The 385-bp PCR product of the methylated and unmethylated GATA-4 were separated by DHPLC at a partial denaturing temperature of 57.1°C and detected by a fluorescence (FL)-detector. The proportion of the methylated GATA-4 in the testing samples was calculated according to the ratio of the peak area for the methylated GATA-4 to the total peak area for both the methylated and unmethylated GATA-4. Genomic DNA of the HCT116 cell line was used as a GATA-4 methylation positive control. PBS was used as a negative control to replace GATA-4/-5 antibody. We regarded GATA-4 and GATA-5 expression as positive when 10% or more cancer cells exhibited GATA-4 and GATA-5 expression.

RESULTS
Aberrant CpG island methylation of the GATA-4 and GATA-5 promoter in primary gastric cancer and their corresponding normal tissues
The promoter methylation status of GATA-4 and GATA-5 in eighty SGCs and their corresponding normal samples were analyzed by MSP. GATA-4 and GATA-5 methylation was observed in 43 and 49 SGCs (53.8% and 61.3%), respectively (Figure 1, Figure 2A and Table 1). To our surprise, these genes were also fre-
quently methylated in the corresponding non-neoplastic samples, 33 for $GATA-4$ (41.3%) and 37 for $GATA-5$ (46.3%), which were only slightly lower than those in SGCs (Figure 2A and Table 1).

To confirm the results of the MSP assay, we established a DHPLC assay to detect the frequency and proportion of the methylated $GATA-4$ in 16 paired-SGC representative samples, in which $GATA-4$ methylation was observed in both SGCs and their corresponding normal tissues by MSP. The methylated and unmethylated $GATA-4$ was completely separated by DHPLC at a partial denaturing temperature of 57.1 ℃ (Figure 3). $GATA-4$ methylation was detected in 15 SGCs (93.8%) and 12 adjacent normal samples (75.0%). The proportion of the methylated $GATA-4$ CpG islands in the DHPLC-positive SGC samples was slightly higher than that in the DHPLC-positive corresponding normal tissues (1.33% ± 0.45% vs 1.10% ± 0.56%, paired $t$-test, $P = 0.054$). The DHPLC results corresponded well with the above MSP results.

**Aberrant CpG island methylation of the $GATA-4$ and $GATA-5$ promoter in gastric dysplasia lesions**

We analyzed the methylation status of these genes in gastric biopsies with low-grade GIN or indefinite for dysplasia in the subjects from Linqu County, a high risk area for stomach cancer in China. Results showed that $GATA-4$ and $GATA-5$ were also frequently methylated in these dysplasia lesions, with strong methylated signals in 46 of 98 (46.9%) and 55 of 104 (52.9%), respectively (Figure 2B and Table 2). More $GATA-4$ and $GATA-5$ methylation was observed in tissues with GIN (57.1% and 69.0%, respectively) than in indefinite for dysplasia...
Figure 3 Chromatogram of the methylated and unmethylated GATA-4 CpG islands by denatured high performance liquid chromatography (DHPLC). The 385-bp PCR product of the methylated and unmethylated GATA-4 were separated by DHPLC at a partial denaturing temperature 57.1°C and detected by a fluorescence (FL)-detector. The proportion of methylated GATA-4 in the tested samples was calculated according to the ratio of the peak area for the methylated GATA-4 to the total peak area for both the methylated and unmethylated GATA-4. The gray arrow points to the peak for the methylated GATA-4 (M) at the retention time 4.5 min; the black arrow points to the peak for the unmethylated GATA-4 (U) at the retention time 3.6 min. Genomic DNA of HCT116 was used as the GATA-4 methylation positive control. The inserted chart represents the dashed line surrounding the area. GATA-4 methylation was detected in the tested sample F0232.

| Table 1  Comparison of GATA-4 and GATA-5 methylation status with clinicopathological characteristics of SGC |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Clinicopathological characteristics | Methylation positive rate (%) | GATA-4 | GATA-5 | GATA-4 | GATA-5 |
| | SGC | N | SGC | N |
| SGC invasion | | | | |
| T1&2 (n = 20) | 45.0 | 35.0 | 45.0 | 45.0 |
| T3 (n = 50) | 54.0 | 42.0 | 68.0 | 48.0 |
| T4 (n = 10) | 70.0 | 50.0 | 60.0 | 40.0 |
| Metastasis | | | | |
| Yes (n = 44) | 52.3 | 31.8 | 61.4 | 36.4 |
| No (n = 36) | 55.6 | 52.8 | 61.1 | 38.9 |
| H. pylori infection | | | | |
| Positive (n = 42) | 61.9 | 45.2 | 61.8 | 50.0 |
| Negative (n = 34) | 41.2 | 38.2 | 55.9 | 44.1 |
| Age (yr) | | | | |
| < 59 (n = 42) | 45.2 | 33.3 | 57.1 | 28.6 |
| ≥ 60 (n = 38) | 63.2 | 50.0 | 65.8 | 65.8 |
| Sex | | | | |
| Male (n = 62) | 51.6 | 41.9 | 61.3 | 51.6 |
| Female (n = 18) | 61.1 | 38.9 | 61.1 | 27.6 |
| Total | | | | |
| n = 80 | 53.8 | 41.3 | 61.3 | 46.3 |

SGC: Sporadic gastric carcinomas; N: Adjacent corresponding non-neoplastic gastric tissue; H. pylori: Helicobacter pylori detected by the 238 rRNA-PCR assay\[4\]; **Analysis for linear trend by EpiInfo 6.0 software, positive rates vs extent of penetration of the stomach wall, P > 0.198 and 0.434, respectively; \*Melani test by EpiInfo 6.0 software, samples from young patients (< 59 years old) vs old patients (≥ 60 years old), P < 0.001; \*Sex vs female, P = 0.074; \*SGCs vs the normal, P = 0.057.

Among the 45 gastric biopsies (27 normal and 18 superficial chronic gastritis), GATA-4 and GATA-5 methylation was only 4/32 (12.5%) and 3/39 (7.7%), respectively (Figure 2C), significantly lower than the methylation in gastric dysplasia and SGCS as well as the adjacent normal samples (P < 0.001).

| Table 3 Correlation of the GATA-4 and GATA-5 methylation status with their protein expression in sporadic gastric carcinomas with IHC |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Protein expression, by IHC | GATA-4 methylation status | GATA-5 methylation status | M | U | P-value |
| | | | | | |
| Positive (+ ~ +++) | 7 | 11 | 0.012 | 5 | 9 | 0.011 |
| Negative (~ -) | 11 | 3 | 16 | 3 | |

HIC: Immunohistochemical staining; ++++: 10% or more cancer cells exhibited a similar positive staining pattern; -: weak. Positive expression in less than 10% cancer cells or a weak staining pattern compared with a non-cancerous area of the same section; M: Methylated; U: Unmethylated; \*Fisher’s exact test by EpiInfo 6.0 software.

The relationship between methylation frequencies of GATA-4 and GATA-5 and clinicopathological parameters including H. pylori infection

As shown in Table 1, an increased trend of GATA-4 methylation was observed in SGCS and the corresponding normal samples with depth of tumor invasion (T1-2, T3, T4), but was not statistically significant. The GATA-5 methylation positive rate in the corresponding normal tis-
sues from elderly and male patients was significantly higher than that from young and female patients, respectively (Table 1).

Moreover, a correlation was observed between GATA-4 (or GATA-5) methylation and H. pylori infection. More GATA-4 and GATA-5 methylation was detected in SGCs and their corresponding normal samples with H. pylori infection than in those without infection, but was not statistically significant (P = 0.071) (Figure 2B). In the gastric dysplasia tissues, particularly in those with low-grade GIN, the GATA-4 methylation positive rate for the patients with H. pylori infection was significantly higher than that in patients without H. pylori infection (15/21 vs 1/7, P = 0.023, Table 2).

In addition, all 4 biopsies with GATA-4 methylation were from H. pylori infected patients (n = 14). No GATA-4 methylation was observed in biopsies from subjects without H. pylori infection (n =18, P = 0.027, two-sides). Of 3 biopsies with GATA-5 methylation, 2 were from H. pylori infected patients (Figure 2C).
DISCUSSION

Epigenetic silencing of GATA-4 and GATA-5 by DNA methylation has been reported in gastrointestinal cancer cell lines and primary carcinomas[5,10,12]. However, the implication of GATA-4 and GATA-5 methylation in the development of gastric cancer is unclear. Hence, we analyzed the methylation status of GATA-4 and GATA-5 in gastric tissues from different kinds of lesions. We found that GATA-4 and GATA-5 methylation was detected in 53.8% and 61.3% of SGCs by MSP, respectively. These results were confirmed by the quantitative DHPLC assay. Furthermore, a significant inverse relationship was observed between methylation status and their protein expression in the gastric samples tested using IHC. These results indicate that GATA-4 and GATA-5 may be frequently inactivated in SGCs by DNA methylation. Moreover, the high prevalence of GATA-4 and GATA-5 methylation in the corresponding normal tissues suggests that these aberrant methylations may be a gastric field-effect, a phenomenon which happens within the whole target regions during carcinogenesis[21].

GATA-4 and GATA-5 were also methylated in 46.9% and 52.9%, respectively, of gastric dysplasia, a precancerous lesion of gastric carcinomas. However, both of these genes were seldom methylated in gastric tissues with or without chronic gastritis [4/32 (12.5%) and 3/39 (7.7%), respectively]. These results indicate that GATA-4 and GATA-5 methylation is an early frequent event during the development of gastric carcinomas.

In addition, we found that the GATA-5 methylation positive rate in the corresponding normal tissues from elderly and male patients was significantly higher than that from young and female patients, respectively. These results are consistent with the higher incidence of gastric carcinoma in males than in females, and the increasing prevalence of gastric carcinomas among elderly subjects.

H. pylori infection is the main cause of chronic atrophic gastritis, which may play an important role in gastric carcinogenesis. A number of tumor-related genes such as p16 could be inactivated by DNA methylation in gastric mucosa lesions with H. pylori infection[22,23]. In the present study, we found that more GATA-4 and GATA-5 methylation in gastric samples from patients with H. pylori infection were detected than in those without H. pylori infection, especially for GATA-4 methylation. The GATA-4 methylation positive rate in the low-grade GIN patients with H. pylori infection was significantly higher than that in patients without H. pylori infection (15/21 vs 1/7, P = 0.023). These results suggest that GATA-4 and GATA-5 methylation could be initiated in the precancerous stage by H. pylori infection.

In addition, among gastric tissues with or without chronic gastritis, all 4 biopsies with GATA-4 methylation were from H. pylori infected patients (n = 14), no GATA-4 methylation was observed in biopsies from subjects without H. pylori infection (n = 18; P = 0.027, two-sides), and of 3 biopsies with GATA-5 methylation, 2 were from H. pylori infected patients. These results again support the hypothesis that H. pylori infection may contribute to epigenetic inactivation of these genes in gastric mucosa.

In conclusion, GATA-4 and GATA-5 methylation was an early frequent field-effect and significantly correlated with the severity of pathological changes during gastric carcinogenesis. H. pylori infection may contribute to GATA-4 and GATA-5 methylation in the human stomach.

COMMENTS

Background

Tumor suppressor genes GATA-4 and GATA-5 are important for development of the stomach during embryogenesis. Epigenetic inactivation of these genes by DNA hypermethylation was previously reported in esophageal and lung cancer.

Innovations and breakthroughs

In the present study, Dr. Wen et al found that aberrant GATA-4 and GATA-5 methylation was also a frequent event in gastric carcinomas and their adjacent tissues. Interestingly, their work demonstrated that epigenetic inactivation of GATA-4 and GATA-5 was observed in about 50% of gastric mucosa samples with epithelial dysplasia, a precancerous lesion in the stomach. However, such a phenomenon was very rare in gastric mucosa biopsies from healthy subjects or patients with chronic gastritis. They also observed that Helicobacter pylori (H. pylori) infection correlated well with GATA-4 and GATA-5 methylation. These results indicate that epigenetic inactivation of GATA-4 and GATA-5 is an early frequent event during gastric carcinogenesis by H. pylori and might be used to screen patients with a high risk of stomach cancer.

Peer review

The authors have tried to show that methylation of GATA-4 and GATA-5 could be important in the oncogenesis of gastric mucosa in patients coming from a Chinese area where gastric cancer is common. They showed that in cancer and adjacent mucosa methylation of these two antioncogenes is common and apparently related mostly with chronic H. pylori infection.

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