Inhibition of Gastric Carcinogenesis by the Hormone Gastrin Is Mediated by Suppression of *TFF1* Epigenetic Silencing

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BACKGROUND & AIMS: Epigenetic alterations have been correlated with field cancerization in human patients, but evidence from experimental models that specific epigenetic changes can initiate cancer has been lacking. Although hormones have been associated with cancer risk, the mechanisms have not been determined. The peptide hormone gastrin exerts a suppressive effect on antral gastric carcinogenesis. **METHODS:** *N*-methyl-*N*-nitrosourea (MNU)-dependent gastric cancer was investigated in hypergastrinemic (INS-GAS), gastrin-deficient ($GAS^{-/-}$), Tff1-deficient ($Tff1^{+/-}$), and wild-type (WT) mice. Epigenetic alterations of the trefoil factor 1 (TFF1) tumor suppressor gene were evaluated in vitro and in vivo. **RESULTS:** Human intestinal-type gastric cancers in the antrum exhibited progressive *TFF1* repression and promoter hypermethylation. Mice treated with MNU exhibited a field defect characterized by widespread *Tff1* repression associated with histone H3 lysine 9 methylation and H3 deacetylation at the *Tff1* promoter in epithelial cells. In MNU-induced advanced cancers, DNA methylation at the *Tff1* promoter was observed. Tumor induction and *Tff1* repression were increased in MNU-treated mice by *Helicobacter* infection. Hypergastrinemia suppressed MNU-dependent tumor initiation and progression in a manner that correlated with gene silencing and epigenetic alterations of *Tff1*. In contrast, homozygous gastrin-deficient and heterozygous *Tff1*-deficient mice showed enhanced MNU-dependent field defects and cancer initiation compared with WT mice. In gastric cancer cells, gastrin stimulation partially reversed the epigenetic silencing in the *TFF1* promoter. **CONCLUSIONS: Initiation of antral gastric cancer is associated with progressive epigenetic silencing of *TFF1*, which can be suppressed by the hormone gastrin.**

Keywords: Gastrin; Gastric Cancer; Epigenetics.

Although both genetic and epigenetic alterations have been characterized in diverse cancers, the critical early steps that result in cancer initiation remain poorly understood. Epigenetic alterations are often detected very early in tumorigenesis in broad regions of histopathologically normal tissue before detection of the incipient tumor,[1,2] a concept referred to as a "field cancerization (defect)."[3] Tumor suppressor genes can be silenced early through hypermethylation of CpG islands frequently found within gene promoter regions, a process that has frequently been associated with changes in histone modifications.[1,2] Growing evidence supports the concept that hormones can modulate cancer risk and regulate the epigenome.[4] However, it remains uncertain whether epigenetic alterations can mediate the relationship between hormones and carcinogenesis. Further, the critical unanswered question is whether these alterations are sufficient to initiate cancer and are not just secondary events.

Although *Helicobacter pylori* infection is the major risk factor for gastric cancer, it is often not sufficient on its own, and a diet of nitrate-rich foods along with tobacco use appear to be significant environmental inducers of gastric cancer.[5] With the development of atrophy and hypochlorhydria during *H pylori*–associated carcinogenesis, dietary nitrates can be converted to endogenous N-nitroso compounds (NOCs) as a result of bacterial overgrowth.[6] NOCs have been found to produce various tumors in animals; however, a causal association between exposure to NOCs and human cancer has not been established.[7] Epidemiologically, increased endogenous formation of NOCs is associated with noncardia cancer risk in *H pylori*–infected patients.[8] We have postulated that NOCs arising from bacterial overgrowth might be responsible for the conversion of metaplasia to dysplasia in human gastric cancer development.[6] The synthetic NOC *N*-methyl-*N*-nitrosurea (MNU) has been used in experimental gastric carcinogenesis.[9] Although MNU is an alkylating agent that can potentially induce formation of DNA adducts and GC → TA transition mutations, only

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; Dclk1, doublecortin-like kinase 1; H3K9, H3 lysine 9; MNU, *N*-methyl-*N*-nitrosourea; NOC, *N*-nitroso compound; PKC, protein kinase C; RT-PCR, reverse-transcription polymerase chain reaction; TFF, trefoil factor; WT, wild-type.

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rare mutations have been observed in NOC-induced gastric tumors of rodents.[9] MNU is also known to modify amino acids in histone proteins, especially histone H3 lysine residues,[10] leading to chromatin remodeling, although the epigenetic effects of NOCs have not been well studied.

Trefoil factor 1 (*TFF1*), a member of the trefoil factor family of peptides, is an antral stomach–specific tumor suppressor gene.[11] *Tff1*$^{-/-}$ mice develop spontaneous antral and pyloric tumors, both adenomas and carcinomas.[12] Another genetic model of antral gastric tumorigenesis, the *gp130*$^{757F/F}$ mouse, shows reduced Tff1 expression.[13] A reduction in *TFF1* gene expression has been observed in about 50% of human distal stomach cancers, and promoter hypermethylation has also been reported.[14–16] Interestingly, a well-defined positive transcriptional regulator of *TFF1* is the peptide hormone gastrin.[17]

Gastrin, a peptide hormone secreted from antral gastrin-expressing cells, was first characterized by its ability to stimulate acid secretion but has also been shown to stimulate proliferation of fundic epithelial cells.[18,19] Although the role of hypogastrinemia as a predisposing factor for antral gastric cancer in patients has not been studied, gastrin knockout (GAS$^{-/-}$) mice develop spontaneous antral tumors under conventional housing conditions.[20] Although hypochlorhydria and bacterial overgrowth are believed to promote cancer in these mice,[21] studies by our group and others suggest that tumorigenesis might be related to *Tff1* repression in gastric antrum.[17,22]

Here we show that progressive *TFF1* epigenetic silencing is one of the mechanisms during carcinogenesis in gastric antrum. Further, we show that the hormone gastrin inhibits *TFF1* repression and thus suppresses antral gastric carcinogenesis.

Materials and Methods

Human Tissues and Mice

Human gastric mucosal tissues, which were collected from 4 pathologic subgroups (Supplementary Tables 1 and 2) in gastric antrum—normal stomach, *H pylori*–positive gastritis, intestinal-type cancer, and preneoplastic adjacent tissue showing intestinal metaplasia—were obtained (Melbourne Health Human Research Ethics Committee approval; Project 2004:176, Kanazawa University Ethics Committee for Human Genome Research approval 2008:174) with informed consent. The animal protocol was approved by the Columbia University Medical Center Institutional Animal Care and Use Committee. INS-GAS male (FVB background), gastrin-deficient, *Tff1*-deficient (B6), and wild-type (WT; FVB and B6) mice were used in this study. Information on cell lines, chemical treatments, *Helicobacter felis* infection, tissue preparation, epithelial cell isolation, immunohistochemistry, quantitative reverse-transcription polymerase chain reaction (RT-PCR), methylation analysis, bisulfite modification, sequencing analysis, chromatin immunoprecipitation (ChIP) assay, and transfection is provided in Supplementary Materials and Methods. All the primers used are shown in Supplementary Table 3.

Results

Progressive Loss of TFF1 *Expression Correlates With Promoter Methylation During Intestinal-Type Carcinogenesis in Human Gastric Antrum*

Gastrin messenger RNA (mRNA) expression was significantly decreased in preneoplastic mucosa with intestinal metaplasia and intestinal-type cancers but was not reduced in *H pylori*–positive mucosa compared with normal mucosa (Figure 1*A*). *TFF1* mRNA expression was significantly decreased in *H pylori*–positive mucosa, preneoplastic mucosa, and cancers compared with normal mucosa (Figure 1*B*). CpG dinucleotides in the *TFF1* proximal promoter were more frequently methylated in *H pylori*–positive gastritis, preneoplastic mucosa, and cancers than in normal mucosa (Figure 1*C*). This shows that *TFF1* promoter hypermethylation is associated with TFF1 repression at the earliest stages of carcinogenesis in gastric antrum. Therefore, although a modest suppression of *TFF1* occurs independently of gastrin levels in *H pylori* infection, there is a clear relationship between *TFF1* epigenetic silencing and loss of gastrin in both intestinal metaplasia and intestinal-type cancers in gastric antrum.

To extend these findings, we investigated whether samples from different pathologic subgroups can be distinguished on the basis of *TFF1* promoter methylation patterns by hierarchical clustering (Figure 1*D*). This analysis broadly partitioned the cancer samples (n = 20) (Supplementary Table 2) across 3 major groups: 2 with *TFF1* hypermethylation containing the majority (50%, 10/20), and one (15%, 3/20) with *TFF1* hypomethylation. A non-adenocarcinoma (choriocarcinoma) was not included in

Figure 1. Epigenetic silencing of *TFF1* during intestinal-type carcinogenesis in human gastric antrum. Quantitative RT-PCR analysis of (*A*) gastrin and (*B*) *TFF1* mRNA levels from 4 indicated pathologic groups. *Y-axis* shows mRNA fold change relative to normal antral stomach. *Black horizontal bars* show mean fold change. **P* < .05. (*C*) A 500–base pair region of the *TFF1* promoter analyzed for methylation. Position of the *TFF1* transcription start site (TSS) is indicated. *TFF1* promoter methylation levels in human gastric tissues determined by EpiTYPER analysis (Sequenom, San Diego, CA). **P* < .05; ****P* < .001. (*D*) Two-way hierarchical cluster analysis of methylation at *TFF1* promoter CpGs (*columns*) in human gastric tissues (*rows*). CpG units in pair 13–14 are shown as combined averages (not resolved on the mass spectrum). Tissue pathology classification is shown on the right vertical axis. CpG methylation ratios: 0 (*green*) to 1.0 (*red*; see *color key*). AGS and MKN28, gastric cancer cell lines.