Helicobacter pylori Infection Promotes Methylation and Silencing of *Trefoil* Factor 2, Leading to Gastric Tumor Development in Mice and Humans

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BACKGROUND & AIMS: Trefoil factors (TFFs) regulate mucosal repair and suppress tumor formation in the stomach. *Tff1* deficiency results in gastric cancer, whereas *Tff2* deficiency increases gastric inflammation. *TFF2* expression is frequently lost in gastric neoplasms, but the nature of the silencing mechanism and associated impact on tumorigenesis have not been determined. **METHODS:** We investigated the epigenetic silencing of *TFF2* in gastric biopsy specimens from individuals with *Helicobacter pylori*-positive gastritis, intestinal metaplasia, gastric cancer, and disease-free controls. *TFF2* function and methylation were manipulated in gastric cancer cell lines. The effects of *Tff2* deficiency on tumor growth were investigated in the $gp130^{F/F}$ mouse model of gastric cancer. **RESULTS:** In human tissue samples, DNA methylation at the *TFF2* promoter began at the time of *H pylori* infection and increased throughout gastric tumor progression. *TFF2* methylation levels were inversely correlated with *TFF2* messenger RNA levels and could be used to discriminate between disease-free controls, *H pylori*-infected, and tumor tissues. Genome demethylation restored *TFF2* expression in gastric cancer cell lines, so *TFF2* silencing requires methylation. In *Tff2*-deficient $gp130^{F/F}/Tff2^{-/-}$ mice, proliferation of mucosal cells and release of T helper cell type-1 (Th-1) 1 cytokines increased, whereas expression of gastric tumor suppressor genes and Th-2 cytokines were reduced, compared with $gp130^{F/F}$controls. The fundus of $gp130^{F/F}/Tff2^{-/-}$ mice displayed glandular atrophy and metaplasia, indicating accelerated preneoplasia. Experimental *H pylori* infection in wild-type mice reduced antral expression of *Tff2* by increased promoter methylation. **CONCLUSIONS: TFF2 negatively regulates preneoplastic progression and subsequent tumor development in the stomach, a role that is subverted by promoter methylation during *H pylori* infection.**

Keywords: Tumor Suppressor; Epigenetics; Stomach Cancer; Trefoil Factor 2.

The trefoil factors (TFF) 1, TFF2, and TFF3 form a family of secreted proteins characterized by a triple loop structure, the trefoil domain.[1] Gastric TFF1 expression is restricted to surface mucus and pit epithelium of the fundus and antrum, whereas TFF2 is expressed in fundic mucus neck cells (MNCs), antrum, and duodenal Brunner's glands.[2,3] TFF3 expression is restricted to intestinal goblet cells.[4] TFF functions include mucus stabilization and barrier protection,[5] regulation of cell migration during wound healing,[6,7] gastric cell differentiation,[8] proliferation, and apoptosis.[9,10]

A body of published evidence shows that *TFF1* is a stomach-specific tumor suppressor gene (TSG). $Tff1^{-/-}$ mice spontaneously develop antral/pyloric tumors,[10] and antral tumors in the $gp130^{Y757F/Y757F}$ ($gp130^{F/F}$) mouse model of gastric cancer show reduced *Tff1* expression.[11] Approximately half of all human gastric cancers show loss of *TFF1* expression[12] through loss of heterozygosity and promoter methylation[13] as well as transcriptional inhibition by regulatory molecules.[14] *TFF1* inhibits gastric cancer cell line growth,[15] blocks G1/S phase cell cycle progression, increases *Retinoblastoma* gene expression,[9] and promotes gastric differentiation,[16] all consistent with a tumor suppressor role in the stomach.

By contrast, the role of *TFF2* in gastric cancer progression is less well understood. *TFF2* expression is rapidly induced in gastrointestinal ulcerative pathologies particularly in regenerating epithelium[17] or after nonsteroidal anti-inflammatory (NSAID) drug treatment.[18] Moreover, in vivo delivery of recombinant TFF2 ameliorates mucosal damage.[19]

Abbreviations used in this paper: 5′-Aza-C, 5′-aza-2′-deoxycytidine; AB/PAS, alcian blue periodic acid-Schiff; Erk, extracellular signal-regulated kinase; IFN, interferon; IL, interleukin; IM, intestinal metaplasia; MNC, mucus neck cell; MPI, month postinfection; mRNA, messenger RNA; PCR, polymerase chain reaction; QRT-PCR, quantitative reverse-transcription polymerase chain reaction; rh, recombinant human; SPEM, spasmolytic polypeptide-expressing metaplasia; Stat, signal transducer and activator of transcription; TFF, Trefoil factor; TSG, tumor suppressor gene.

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TFF2 gives its former name to a mucus metaplasia, of mainly fundic origin known as *spasmolytic polypeptide expressing metaplasia* (SPEM).[20] Whereas SPEM is associated with *Helicobacter pylori*-dependent fundic gland atrophy[21] and may be a precursor to gastric cancer,[22] *TFF2* is dispensable for the development of SPEM-like metaplasia.[23] In addition, *Tff2*-deficient mice display only subtle alterations in mucosal proliferation, parietal cell activation, and reduced reparative function.[18]

An anti-inflammatory role for *Tff2* has recently been described. *Tff2*-deficient mice display immune deregulation and are more susceptible to dextran sodium sulphate-induced colitis and *Helicobacter felis*–induced gastritis, developing more severe inflammatory pathology than wild-type controls.[24,25] Furthermore, *H pylori*-infected *Tff2*-deficient mice develop more advanced premalignant lesions of atrophy, metaplasia, and dysplasia than wild-type littermates.[26] Therefore, in addition to immune modulation, *TFF2* may impose growth restraint and limit neoplastic progression in the context of chronic inflammation.

Here, we demonstrate that loss of *TFF2* expression occurs during the progression of human intestinal-type gastric cancer via the acquisition of aberrant promoter methylation. To determine the likely functional impact of *TFF2* loss (via promoter methylation) on gastric tumor growth, we have employed a genetic approach to disrupt *Tff2* function in murine gastric cancer. The $gp130^{Y757F/Y757F}$ ($gp130^{F/F}$) mouse is a model of gastric tumorigenesis that develops antral tumors with histopathologic similarities to human intestinal-type gastric cancer.[27] We show that genetic *Tff2* deficiency substantially accelerates tumor growth in $gp130^{F/F}$ mice, primarily via further loss of gastric TSGs and misexpression of hormones and cytokines that promote preneoplastic fundic atrophy and chronic gastritis. These data support the view that *TFF2* may act locally to suppress preneoplastic change and subsequent gastric tumor development, a role that is ultimately subverted by epigenetic silencing after *H pylori* infection.

Materials and Methods

Mice

$Tff2^{+/-}$ and $gp130^{F/+}$ heterozygous mice[11,18] were crossed to obtain wild-type, $Tff2^{-/-}$, $gp130^{F/F}$, and compound mutant $gp130^{F/F}/Tff2^{-/-}$ mice and maintained on a C57BL6/129-Sv mixed background. Age-matched littermate controls were used in all experiments. Wild-type (C57BL6) mice were infected with *H pylori* Sydney strain 1 (SS1) as described.[26] Experiments were performed in accordance with either the Royal Melbourne Hospital (approval No. 010/2005) or Murdoch Children's Research Institute Animal Ethics Committee (approval No. A583).

Human Tissue Sources

Gastric mucosal tissue was obtained from *H pylori*-positive individuals and disease-free controls as described.[28] Gastric cancers and matched preneoplastic adjacent to cancer tissues were obtained with informed consent (approval No. 174.2008; Kanazawa University Ethics Committee for Human Genome Research).

Morphometric and Histologic Analysis

Mouse stomachs were removed, pinned, and photographed for macroscopic morphometry. Paraffin sections (4 μm) stained with H&E or alcian blue periodic acid-Schiff (AB/PAS) were used for microscopic morphometry and histologic assessment. Morphometric analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij/index.html; National Institutes of Health, Bethesda, MD). The gastric mucosal area and length were outlined with the calibrated software drawing tool at the appropriate magnification. Inflammation was assessed by microscopy in a blinded fashion on AB/PAS and H&E-stained sections. Antral tumor tissues were analyzed with a minimum of 3 separate samples per animal cut and scored according to the density of inflammatory cells from minimum = 0 to maximum = 3[29] including infiltration around the muscularis mucosa, submucosal inflammation, and intramucosal infiltration of the tumors.

Mammalian Cell Culture

AGS and MKN28 gastric cancer cell lines were maintained in RPMI 1640 Glutamax I growth medium, 10% fetal bovine serum, 2 mmol/L nonessential amino acids, 50 IU/mL penicillin, 50 μg/mL streptomycin (all Invitrogen, Carlsbad, CA), at 37°C in a humidified incubator with 5% CO_2/air.

For proliferation analysis, AGS cells were serum starved overnight then seeded in 6-well plates at 2×10^5 cells/well in growth medium supplemented with 0.2% fetal bovine serum. Recombinant human (rh) TFF2 was used at 50 μg/mL. Viable cell counts were performed by 0.4% trypan blue dye exclusion on a hemocytometer. For apoptosis, cells were stained with annexin V-Alexa Fluor 488 conjugate (Invitrogen Molecular Probes, Carlsbad, CA) and propidium iodide according to the manufacturer's protocols. Apoptotic (annexin V positive, propidium iodide negative) cells (10,000 events) were resolved on an LSRII flow cytometer (BD Biosciences, San Diego, CA) and analyzed using FACS Diva software (BD Biosciences). For genome demethylation studies, AGS and MKN28 cells were plated in 25-cm flasks at 5×10^5 cells and allowed to attach overnight. Cells were treated with 10 μmol/L 5′-aza-2′-deoxycytidine (5′-Aza-dC) dissolved in dimethyl sulfoxide (Sigma) or dimethyl sulfoxide alone for 72 hours, with fresh 5′-Aza-dC added at 24-hour intervals.

Quantitative DNA Methylation Analysis

Genomic DNA was isolated from tissue using the DNeasy tissue kit (Qiagen, Hilden, Germany) or using Trizol (Invitrogen) and 200–500 ng of each sample was bisulphite converted using Zymo EZ DNA methylation