# BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

## *Helicobacter pylori* Causes Epigenetic Dysregulation of *FOXD3* to Promote Gastric Carcinogenesis

ALFRED S. L. CHENG,[1] MAY S. LI,[1] WEI KANG,[3] VICTORIA Y. CHENG,[2] JIAN–LIANG CHOU,[6] SUKI S. LAU,[1] MINNIE Y. GO,[2] CHING C. LEE,[5] THOMAS K. LING,[5] ENDERS K. NG,[4] JUN YU,[1,2] TIM H. HUANG,[7] KA F. TO,[1,3] MICHAEL W. CHAN,[6] JOSEPH J. Y. SUNG,[1] and FRANCIS K. L. CHAN[1,2]

[1]*Institute of Digestive Disease,* [2]*Departments of Medicine and Therapeutics,* [3]*Anatomical and Cellular Pathology,* [4]*Surgery, and* [5]*Microbiology, The Chinese University of Hong Kong, Hong Kong SAR, China;* [6]*Department of Life Science, National Chung Cheng University, Chia-Yi, Taiwan, Republic of China; and* [7]*Department of Molecular Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas*

**See editorial on page 22.**

**BACKGROUND & AIMS:** Deregulation of forkhead box (Fox) proteins, an evolutionarily conserved family of transcriptional regulators, leads to tumorigenesis. Little is known about their regulation or functions in the pathogenesis of gastric cancer. Promoter hypermethylation occurs during *Helicobacter pylori*–induced gastritis. We investigated whether the deregulated genes contribute to gastric tumorigenesis. **METHODS:** We used integrative genome-wide scans to identify concomitant hypermethylated genes in mice infected with *H pylori* and human gastric cancer samples. We also analyzed epigenetic gene silencing in gastric tissues from patients with *H pylori* infection and gastritis, intestinal metaplasia, gastric tumors, or without disease (controls). Target genes were identified by chromatin immunoprecipitation microarrays and expression and luciferase reporter analyses. **RESULTS:** Methylation profile analyses identified the promoter of *FOXD3* as the only genomic region with increased methylation in mice and humans during progression of *H pylori*–associated gastric tumors. *FOXD3* methylation also correlated with shorter survival times of patients with gastric cancer. Genome demethylation reactivated FOXD3 expression in gastric cancer cell lines. Transgenic overexpression of *FOXD3* significantly inhibited gastric cancer cell proliferation and invasion, and reduced growth of xenograft tumors in mice, at least partially, by promoting tumor cell apoptosis. FOXD3 bound directly to the promoters of, and activated transcription of, genes encoding the cell death regulators CYFIP2 and RARB. Levels of *FOXD3*, *CYFIP2,* and *RARB* messenger RNAs were reduced in human gastric tumor samples, compared with control tissues. **CONCLUSIONS: FOXD3-mediated transcriptional control of tumor suppressors is deregulated by *H pylori* infection–induced hypermethylation; this could perturb the balance between cell death and survival. These findings identify a pathway by which epigenetic changes affect gastric tumor suppression.**

*Keywords:* Stomach Cancer; CpG-Island Methylation; Gene Expression; Inflammation.

*Helicobacter pylori* (*H pylori*) is a class I carcinogen in gastric cancer,[1] the second most deadly malignancy worldwide.[2] Chronic *H pylori* infection induces gastric cancer through stages of chronic gastritis, atrophy, intestinal metaplasia (IM), and dysplasia.[3] We and others have carried out randomized trials to test the effects of *H pylori* eradication in populations with a high incidence of gastric cancer.[4–8] Although prophylactic eradication of *H pylori* after endoscopic resection of early gastric cancer has been shown to prevent development of metachronous gastric cancer,[4] curing *H pylori* infection alone does not influence the incidence of de novo cancer.[5–8] More importantly, the benefit of *H pylori* eradication in the prevention of cancer development was confined to individuals with no premalignant lesions, ie, atrophy, IM, or dysplasia on baseline examination.[8] Once IM has developed in the stomach, there is a >6-fold increase in risk of gastric cancer,[9] suggesting that molecular alterations are "imprinted" in the gastric epithelium genome of premalignant lesions. However, the identity of such genomic defects remains largely unknown.

Genetic and epigenetic mechanisms are intertwined in oncogenic circuits of molecular alterations and pathway dysregulation.[10,11] In contrast to genetic alterations, epigenetic changes, such as DNA methylation and histone modifications, influence the phenotypic outcomes of a genome without changes in the underlying DNA sequences. Such reversible nature provides great opportunities for the development of epigenetic targets or markers for treatment, diagnosis,

*Abbreviations used in this paper:* ChIP, chromatin immunoprecipitation; FOXD3, forkhead box D3; IM, intestinal metaplasia; mRNA, messenger RNA; NF, nuclear factor; PARP, poly (ADP-ribose) polymerase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SS1, Sydney Strain 1; TSS, transcriptional start site.


0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2012.10.002

and prognosis of various cancers.[11] Stomach frequently shows aberrant methylation of DNA in epithelial cells because of its accessibility to exogenous toxic agents such as *H pylori*.[12,13] We and others have shown that *H pylori* infection in patients was associated with promoter methylation of genes related to gastric cancer initiation and progression.[14–18] Intriguingly, eradication of *H pylori* could reduce gene methylation in patients with gastritis but not IM.[16–18] Taken together, these studies suggest that aberrant DNA methylation accumulated in the premalignant lesions can contribute to the "point of no return" in gastric carcinogenesis. Although genome-wide screening has identified panels of genes undergoing aberrant promoter methylation in gastric cancer,[19] this analysis alone cannot distinguish between causal epigenetic alterations from "bystander changes." A recent study has demonstrated that *H pylori* infection reduces tumor suppressor gene expression by increased promoter methylation.[20] However, the functional significance of *H pylori* epigenetically deregulated genes in gastric carcinogenesis remains unclear.

Composed of 17 subfamilies (A to R), forkhead box (Fox) genes are evolutionarily conserved transcriptional regulators controlling a wide spectrum of biological processes, including metabolism, differentiation, proliferation, apoptosis, and migration.[21] Foxd3 (or Genesis) was first identified in early mouse embryonic development.[22,23] Although deregulation of certain Fox genes plays a crucial role in the carcinogenesis,[21] the regulation and functions of FOXD3 in cancer remain largely unknown.

Using integrated genome-wide analysis, we delineated an aberrant DNA methylation initiated by chronic *H pylori* infection that affects the prognosis of gastric cancer patients. We have shown that promoter hypermethylation of *FOXD3* is an early epigenetic event that correlates with progressive loss of expression in the multistep gastric carcinogenesis. We further determined the tumor-suppressive functions of FOXD3 in gastric cancer, which can be mediated by its direct transcriptional targets, *CYFIP2* and *RARB*. These tumor suppressors are concordantly down-regulated with FOXD3 during gastric cancer progression, demonstrating the clinical relevance of our findings.

## Materials and Methods

Details for chromatin immunoprecipitation (ChIP), ChIP-chip, functional assays, DNA methylation, and gene expression analyses are contained in the Supplementary Material.

### *Mice*

All procedures were approved by the Animal Experimentation Ethics Committee of Chinese University of Hong Kong. Male C57BL/6 mice were gavaged with *H pylori* Sydney Strain 1 (SS1) or broth control, as described previously.[24] The mice were sacrificed after 40 weeks. Stomach tissues were collected for histological examination, *H pylori* confirmation, DNA methylation, and gene expression analyses.

### *Human Tissue Sources*

Gastric mucosal tissues were obtained with written consent from uninfected normal stomach, *H pylori*–positive gastritis, IM, gastric cancer, and matched adjacent noncancer tissues, as described previously (Supplementary Tables 1 and 2).[25] The study protocol was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee.

### *Methylated-DNA Capture (MethylCap)-Microarray*

Genomic DNA was extracted and sonicated into fragments of 200 to 600 base pairs. Methylated DNA was isolated with affinity purification[26] using MethylMiner Methylated DNA Enrichment kit (Invitrogen, Carlsbad, CA), amplified and hybridized onto promoter arrays (Agilent, Santa Clara, CA) as described previously.[27] Replicate dye-swap experiments were conducted and analyzed by ChIP Analytics software (Agilent).

### *Microarray-Based Methylation Assessment of Single Samples*

One microgram DNA of human gastric cancer tissue was subjected to microarray-based methylation assessment of single samples,[28] hybridized onto human CpG 12.1K arrays (UHN Microarray Centre) and analyzed as described previously.[29] Quantile normalization and Predication Analysis for Microarrays were then performed to identify differentially methylated genes. The microarray data have been deposited in the Gene Expression Omnibus database (accession number: GSE39600).

### *Statistical Analysis*

The differences in DNA methylation and messenger RNA (mRNA) levels between 2 groups were analyzed using Mann-Whitney *U* test. We used Kaplan-Meier analysis to assess survival in relation to methylation status. High *FOXD3* methylation level was classified based on highest tertile. Between-group difference was determined by log-rank test. Student *t* test was used to compare difference between sample means. All tests were 2-tailed and $P < 0.05$ was considered significant.

## Results

### *Integrative Epigenomics Analysis Reveals Common Hypermethylated Gene for* H pylori *Infection and Gastric Cancer Prognosis*

To identify *H pylori* infection–associated hypermethylated genes on a genomic scale, we carried out MethylCap-microarray[26] using gastric tissues from mice infected with *H pylori* SS1 or broth control for 40 weeks (Supplementary Figure 1*A*). Using promoter arrays containing approximately 19,000 best-defined mouse genes, we found that 317 gene promoters were significantly hypermethylated in *H pylori*–induced gastritis tissues compared with uninfected controls in both dye-swap replicates (Figure 1*A* and Supplementary Table 3). Gene ontology analysis revealed significant enrichment of genes related to cancer development, namely regulation of transcription and cell differentiation (Supplementary Figure 1*B*). Most of the cell differentiation–related genes, including *Hand1*[15] and *Pcdh10*,[25] (Supplementary Figure 1*C*) are silenced by promoter methylation in human gastric cancer or other malignancies, as reported previously (Supplementary Figure 1*D*), supporting the clinical relevance of the novel *H pylori*–hypermethylated genes.

To identify hypermethylated genes that may correlate with survival, we compared CpG-island methylation pro-