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Gastric Cancer Prevention by Demethylation

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Abstract

Niwa *et al*. report in this issue that treatment with the DNA demethylation agent 5-aza-2′-deoxycytidine decreases the incidence of gastric cancers in an animal model of *Helicobacter pylori*-promoted gastric cancer. This provocative study underscores the importance of changes in DNA methylation that contribute to the origin of inflammation-related cancers. The findings also raise the exciting possibility of cancer prevention by altering DNA methylation events early during tumorigenesis.

Worldwide, an estimated 16% of cancers, accounting for approximately 2 million new cases per year, are related to infectious agents, including hepatitis B and C viruses, human papilloma virus, Epstein Barr virus and *Helicobacter pylori (H. pylori*; ref. 1). The latter agent, a spiral bacterium adapted to reside in the human stomach, promotes the development of gastric B-cell lymphoma and gastric adenocarcinoma, a disease that will kill an estimated 10,990 Americans in 2013 (2). Annually, approximately 660,000 new gastric cancer cases worldwide are related to *H. pylori* infection (1).

Multiple mechanisms have been proposed for the promotion of gastric cancer by *H. pylori*. The *cag* pathogenicity island, a 40-kb locus present in some strains, is associated with an increased risk of distal gastric cancer, compared to strains lacking the *cag* island (3, 4). The *cagA* gene, found at the terminal region of the island, encodes a CagA oncoprotein, which is injected into gastric epithelial cells via the bacterial Type IV secretion system, whereupon CagA becomes phosphorylated by Src family kinases and activates SHP-2 tyrosine phosphatase, disrupting cell signaling pathways. Phosphorylated CagA binds to PAR1 (5), Crk adaptor proteins, c-Met, and ZO-1. PAR1 participates in the establishment and maintenance of epithelial cell polarity, while Crk proteins are involved in regulation of the actin cytoskeleton, cell proliferation and migration (6). The hepatocyte growth factor receptor c-Met affects cell motility, and CagA deregulates c-Met signaling (7). CagA also binds to the tight junction scaffolding protein ZO-1 and alters the apical-junctional complex (8). Which one or more of these functions may be responsible for the oncogenic effects of CagA is not clear, but gastrointestinal and other malignancies develop when CagA is expressed in transgenic mice, but only if CagA tyrosine phosphorylation capability is maintained (9).

In addition to these CagA-dependent functions, *H. pylori* infection exerts more general influences related to oxidative and nitrosative effects that may contribute to carcinogenesis. Chronic inflammation is accompanied by an influx of neutrophils and macrophages, which

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generate and release reactive oxygen species and reactive nitrogen intermediates, leading to further increased inflammation and DNA damage. *H. pylori*'s secreted virulence factor, HP-NAP (neutrophil activating protein), induces the assembly of neutrophil NADPH oxidase components from the cytoplasm and membranes. Although in principle, assembled NADPH complexes can be targeted to either phagosomes or the extracellular space, in the case of *H. pylori* infection, complexes are preferentially targeted to the latter location (10), leading to a release of superoxide that enhances inflammation. This effect is advantageous to *H. pylori*, as local tissue damage is enhanced, which releases nutrients. Macrophages, in addition to their role of producing IL-12 that activates Th1 cells, also function as effector cells by generation of nitric oxide (NO) catalyzed by inducible nitric oxide synthase (iNos). Following *H. pylori* infection, iNos is upregulated in human gastric mucosae (11), and this effect is accompanied by increases in the production of the DNA adducts 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG, also called 8-OHdG; ref. 12). *H. pylori* infection also generates H_2O_2 within gastric epithelial cells by upregulating spermine oxidase, the enzyme that oxidizes spermine to release H_2O_2, leading to DNA damage detectable as 8-OHdG (13). Mispairing of 8-OHdG during DNA replication may lead to G > T transversion mutations (14).

Another consequence of *H. pylori* infection related to carcinogenesis is alteration in patterns of DNA methylation in gastric epithelial cells. Aberrant methylation of the *CDH1* promoter has been associated with the presence of *H. pylori* infection in dyspeptic patients (15). Subsequent studies reported aberrant methylation of other genes, including *LOX, HAND1, THBD, HRASLS, FLNC, ARC, CDKN2A*, and *TWIST1*, associated with *H. pylori* infection (16, 17). Aberrant methylation has been found to be partially reversible after *H. pylori* is eradicated (18, 19). Using the Mongolian gerbil *(Meriones unguiculatus)* model of *H. pylori*-induced gastric cancer, Niwa and colleagues in a previous study proposed that inflammation, rather than the *H. pylori* per se, promoted hypermethylation, because animals treated with the immunosuppressant cyclosporine A did not undergo hypermethylation of the monitored genes, even though levels of *H. pylori* remained constant (20).

In this issue, the same investigators now report that treatment of gerbils with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) reduces the incidence of neoplasia in an animal model of inflammation-promoted gastric cancer (treatment of gerbils with both *H. pylori* and the carcinogen *N*-methyl-*N*-nitrosourea, or MNU; ref. 21). Following 5-aza-dC treatment, cancer incidence declined from 55.2% to 23.3%. Evaluation of methylation levels of 6 methylation-prone promoters revealed a partial reversal in methylation following the use of 5-aza-dC, while global methylation levels declined slightly. Previously, the same investigators had measured increases in expression of *Il1b, Nos2* and *Tnf* accompanying *H. pylori* infection in this model (20). In the current article, they report that in gerbils treated with 5-aza-dC, the expression of *Il1b* and *Nos2* in gastric mucosae declined to 42% and 58% of untreated levels. In contrast, *Tnf* expression increased to 187% of untreated levels. Notably, these dysregulatory changes in response to 5-aza-dC occurred without a significant change in the levels of mononuclear or polymorphonuclear cell infiltration. Regarding side effects of treatment, testicular atrophy was observed, but no histological changes were detected in the small intestine, liver or kidneys. The investigators note that this is the first report of prevention of an inflammation-induced cancer by a demethylation agent.

Alterations in DNA methylation are likely to be key early steps in the process of carcinogenesis. According to the Epigenetic Progenitor Model of Cancer (22), tumors arise from epigenetic disruption of progenitor or stem cells, and epigenetic changes, especially alterations in DNA methylation, may lead to aberrant inactivation of tumor suppressor genes and activation of oncogenes. Epigenetic changes are polyclonal, which is consistent with the field defect, long noted in gastric and other tumor types (23). Therefore, it is reasonable that

prevention efforts directed at the primary epigenetic dysfunction may be effective. Clinically, 5-aza-dC (decitabine), is now being used either alone or in drug combinations for the treatment of various malignancies, including non-small cell lung cancer (24), acute myeloid leukemia (25), and myelodysplastic syndrome (26). Considerations of costs and side effects are significant challenges to the prophylactic use of these drugs in the near future. Despite these limitations, the ramifications of the Niwa study inspire hope for better drugs or therapies for reversal of methylation changes sustained during the precancerous process.

A possible mechanism for inflammation-induced dysregulation of DNA methylation has been described by O'Hagan *et al.* as DNA repair gone awry (27). Following DNA damage, histone H2AX becomes serine 139 phosphorylated as γ–H2AX (28) and stabilizes interaction of repair proteins with chromatin. O'Hagan *et al.* found increased avidity of binding of DNA methyltransferase 1 (DNMT1) and the NAD+-dependent class III histone deacetylase (SIRT1) to chromatin following treatment of human embryonic carcinoma cells with H_2O_2. DNA damage produced by ionizing radiation or ultraviolet light did not reproduce the effect. Immunoprecipitation experiments revealed that DNMT1 and SIRT1 became part of a complex containing DMNT3B and members of the polycomb repressive complex 4 (EZH2, SUZ12, and EED2). The multiprotein complexes (or "silencing complexes") preferentially targeted GC-rich regions, such as those found in some promoters. Those GC-rich regions were, as might be expected, enriched in 8-oxo-dG. Following H_2O_2 treatment, a set of genes with high transcription rates had those rates reduced, in association with binding of the silencing complex. In contrast, in the same short time frame (30 minutes following treatment), a set of genes with low basal transcription rates showed increased DNA methylation associated with the binding of the complex. Tumor-associated increases in promoter methylation occur more frequently in genes with low basal transcription rates (29). To test whether the same avid binding of SIRT1 and EZH2 occurred *in vivo*, the investigators examined a mouse model of an inflammation-related colon cancer (Min mice infected with *Bacteroides fragilis*) and found tighter binding of those two proteins in the distal portion of the mouse colon, where inflammation is greater. Co-immunoprecipitation experiments also demonstrated more DNMT1 complexed with EZH2 in the tissue where the severity of inflammation was greater. Low expression genes with CpG island-containing promoters (including *Fbn1, Sez6l, Sfrp5* and *Sox17,* which undergo tumor-specific DNA methylation) showed enrichment of EZH2 and DMNT1 at the promoter CpG islands from the inflamed tissue. These results are consistent with a model of tumor-specific DNA methylation resulting from or secondary to a process adapted to repair DNA damage arising from oxidative stress, especially in GC-rich regions like CpG islands. High expression genes seem somehow protected against DNA methylation, whereas low expression genes are not and consequently sustain such methylation.

Work from our group has indicated that the presence of the *cagA* gene in the infecting *H. pylori* strain may also be related to methylation effects in the gastric epithelium of the host. *H. pylori* strains isolated from subjects residing in regions of high and low risk for gastric cancer were characterized for the presence of *cagA*. Gastric biopsy DNA from the corresponding subjects was analyzed for aberrant methylation at 4 candidate gene promoters (*RPRM, APC, MGMT* and *TWIST1*). In a multivariate analysis, the presence of *cagA* was independently associated with elevated levels of methylation at promoters of *RPRM, APC* and *MGMT* (30). Whether this association is a result of the greater inflammatory response induced by *cagA*-positive strains (31) or to one of the other effects of *cagA* described above is not yet clear.

The current study by Niwa *et al.* is another example of the utility of the Mongolian gerbil model for *H. pylori*-promoted gastric cancer, as this model recapitulates most closely the

human disease, among small animal models. Wild type mice do not develop gastric cancer after inoculation with *H. pylori*, and even transgenic models such as INS-GAS mice do not fully reproduce human gastric cancer as well as the gerbil. Future efforts to understand inflammation-promoted gastric cancer may include sequencing of the gerbil genome, as well as the development of species-specific reagents. Studies such as the current investigation also highlight the importance of studying microbial and host constituents in a conjoined fashion rather than in isolation. A holistic approach will permit more detailed insights into the complex pathways that lead to gastric adenocarcinoma.

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