In 1992, we addressed this issue with a sensitive PCR detection system for the *Bam*HI W/Z rearrangement in EBV-positive HD.[3] Genomic DNA was purified under sterile conditions from fresh frozen lymph node biopsies of 21 adult Swiss patients. Sixteen patients suffered from EBV-positive HD (LMP1 expressing Reed-Sternberg cells) and five from EBV-associated angioimmunoblastic lymphadenopathy. All biopsy samples contained a high number of EBV-copies, in particular 15 samples $\geq 10^4$ copies per 1 $\mu$g DNA and six samples with $10^3$ copies per 1 $\mu$g DNA, when tested with a semi-quantitative graduated dilution method.[4] The cell line P3J-HR-1, clone HH543–5 (a kind gift from Dr. G. Miller, Yale University), was used as a positive control for *Bam*HI W/Z rearrangements.[3] The results were verified by Southern blotting with subsequent specific hybridization including the positive control.

In comparing our findings in EBV-positive HD (all 16 HD cases negative for *Bam*HI W/Z rearrangements) with the results of Gan and colleagues[1] (32% positive), it should be noted that this significant difference occurred even though both groups used a very sensitive PCR detection method; and samples with a high EBV-copy number. What then could make up for this difference? Could it be the geographic origin of the samples? We don't think so, because another polymorphism within the EBV genome, the 30-bp LMP1-deletion variant, is observed at equal frequency (59 to 75%) in infectious mononucleosis or EBV-associated tonsillar hyperplasia of children and adolescents from Brazil, North America, and Switzerland.[5,6]

What remains, therefore, is the difference of age at diagnosis of HD, which was 40 years (mean) in our series. In adults, a putative loss of defective EBV genomes harboring *Bam*HV W/Z rearrangements over time might be a consequence of a still immunocompetent organism in childhood and early adulthood, and in the years before development of HD which is associated with impaired T-cell immunity.[7]

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## *Gene Methylation in Non-Neoplastic Mucosa of Gastric Cancer: Age or* Helicobacter pylori *Related?*

To the Editor-in-Chief:

We read with interest the article of Waki et al[1] on the promoter methylation status of *E-cadherin,* h*MLH1*, and *p16* genes in non-neoplastic gastric epithelia. We have also recently studied the methylation of the *E-cadherin* gene in non-neoplastic mucosa of patients with gastric cancer.[2] The methylation of *E-cadherin* was assessed by two different primer sets as designated by Herman et al[3] and Graff et al[4] to confirm the specificity. We observed that *E-cadherin* methylation was associated with age, the presence of chronic gastritis, and *Helicobacter pylori* (*H. pylori*) on univariate analysis. However, *H. pylori* infection was the only independent factor associated with *E-cadherin* methylation on multivariate analysis.

Hypermethylation at the promoter region of tumor suppressor gene has been shown to be an important mechanism in gene silencing. However, gene-acquired methylation may not necessarily be associated with neoplastic transformation. Toyota et al[5] proposed that two types of methylation might exist: type A methylation (such as the *estrogen receptor* gene) which is age-related and may be present in normal mucosa and type C methylation which is cancer-related and not present in normal mucosa. Furthermore, recent studies have shown that gene methylation may be present in non-neoplastic colorectal mucosa in patients with inflammatory bowel disease,[6,7] esophageal mucosa in patients with Barrett's esophagitis,[8,9] and in liver tissues in chronic hepatitis.[10] In the stomach, methylation at *THBS-1* and *TIMP-3* in chronic gastritis was found to be present in 10.1% and 14.5% of cases respectively.[11] These findings suggested that gene methylation could be a result of chronic inflammation.

In the stomach, the interplay between age and chronic inflammation is complicated by the presence of *H. pylori* infection. The prevalence of *H. pylori* increases with age. In Western countries, about 50% of people over 60 years of age are infected, but only 20 to 30% below the age of 30 are infected.[12,13] In addition, the presence of *H. pylori* is almost invariably associated with gastritis. In a meta-

analysis, Blaser[14] documented that 75% to 100% of symptomatic patients or asymptomatic volunteers with endoscopically proven gastritis were colonized with the organism. When a person is infected with *H. pylori*, a superficial gastritis results within hours or days and can progress to chronic gastritis. The prevalence of *H. pylori* is higher in China, Japan, and Korea than in Western countries. This fact might explain why *E-cadherin* methylation was absent in non-neoplastic gastric mucosa when tested in Western patients,[3,15] but present in studies performed in patients from Japan, Hong Kong, and Korea.[1,2,11] Hence, the issue of whether methylation in the non-neoplastic gastric mucosa is due to aging, chronic gastritis, or *H. pylori* infection should be addressed carefully.

Experimental data from *in vitro* studies support our contention that *E-cadherin* methylation might be related to *H. pylori* infection. El-Omar et al[16] reported that interleukin-1$\beta$ polymorphism that led to up-regulation of interleukine-1$\beta$ with *H. pylori* infection was associated with increased risk of gastric cancer. Furthermore, Hmadcha et al[17] found that interleukin-1$\beta$, through the production of nitric oxide and the subsequent activation of DNA methyltransferase, might induce gene methylation. It is thus possible that *H. pylori* induces methylation through the production of interleukin-1$\beta$.

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## *Author's Reply:*

The authors appreciate the comments by Dr. Chan et al on our paper describing gene promoter methylation in non-neoplastic gastric epithelia.[1] A number of tumor suppressor and tumor-related genes exhibited promoter methylation in both neoplastic and non-neoplastic gastric epithelia, although certain gene methylation was found to be cancer-specific.[2,3] Such differences in methylation patterns among genes may correspond to type A (aging-specific) and type C (cancer-specific) methylation previously described for colorectal and gastric epithelial cells.[4,5] Type A methylation arises as a function of age in normal cells, potentially affecting genes that regulate the growth and/or differentiation of these cells, and could account, in part, for the hyperproliferative state that is thought to precede tumor formation.[4] In contrast, type C methylation affects only a subset of tumors, which then evolve along a global hypermethylation pathway.[4] However, *hMLH1* methylation, once thought to be cancer-specific,[4] was found to be a common age-related event in normal colonic cells when the entire *hMLH1* promoter ~700-bp region was analyzed.[6] *hMLH1* methylation is partial in normal colonic cells and increases with age, spreading to reach a threshold, and ultimately shutting down protein expression.[6] Therefore, these contradictory results might be due to the analysis of different CpG sites in these studies. If critical CpG sites for each indication of gene silencing are more precisely analyzed, age-related

**Table 1.** Frequencies of Gene Promoter Methylation in Nonneoplastic Gastric Epithelia

| Gene | ≦ 31 years of age | | | ≧ 43 years of age | | |
|---|---|---|---|---|---|---|
| | U ($n$ = 7) | M ($n$ = 11) | L ($n$ = 7) | U ($n$ = 23) | M ($n$ = 25) | L ($n$ = 22) |
| *APC* | 57% | 36% | 57% | 91 | 96% | 95% |
| *DAP-kinase* | 0% | 0% | 0% | 78% | 80% | 68% |
| *E-cadherin* | 0% | 0% | 0% | 78% | 76% | 64% |
| *p16* | 0% | 0% | 0% | 30% | 16% | 18% |
| *RUNX3* | 0% | 0% | 0% | 4% | 4% | 32% |
| *RASSF1A* | 0% | 0% | 0% | 4% | 12% | 5% |
| *hMLH1* | 0% | 0% | 0% | 4% | 0% | 14% |
| *GSTP1* | 0% | 0 | 0% | 0% | 0% | 0% |

RT-PCR analysis on IFN-γ-inducible expression of promoter III-derived (Pro III) and promoter IV-derived (Pro IV) CIITA (38 cycles of PCR) and GAPDH (28 cycles of PCR) in small cell lung cancer (SCLC), neuroblastoma (NB), and non-SCLC cell lines. Cultured cancer cells were treated with 500 U/ml IFN-γ for 24 hours.

U, upper third portion of the stomach; M, middle third portion of the stomach; L, lower third portion of the stomach.

methylation may be found to be cancer-specific. This hypothesis is supported by the observation that *DAP-kinase* methylation was present in virtually every tumor and normal gastric and colorectal cell when the edge of a CpG island was examined, yet on analysis of the central region of the CpG island, the methylation was determined to be a more infrequent, cancer-specific phenomenon.[7] Thus, the differences of methylation patterns may not provide any evidence for the existence of any CpG island methylator phenotype, and some (or cancer cells) are simply more hypermethylated than others (or non-neoplastic cells).[8] Alternatively, age-related methylation may not be immediately oncogenic, but gradually spreads to inactivate gene function. In fact, age-related methylation of several tumor suppressor and tumor-related genes was observed in the small intestine where tumor evolution is extremely rare.[3]

Because several factors may modulate age-related methylation, such as exogenous carcinogens, endogenously generated reactive oxygen species, and genetic differences in individual susceptibility,[9] *Helicobacter pylori* (*H. pylori*) infection and resultant gastritis may accelerate this process. We further explored methylation analysis in non-neoplastic gastric epithelia obtained at autopsy from 11 non-gastric cancer patients younger than 32 years old (range, 0 to 31 years; average, 14.0 years, excluding stillborn infants) and 25 non-gastric cancer patients greater than 42 years old (range, 43 to 87 years; average, 67.9 years) (Table 1). Methylation was absent in younger individuals, except in *APC* (promoter 1A) (Table 1). Methylation of one of the promoters (promoter 1A) is not oncogenic because the other (promoter 1B) is protected from methylation and thus *APC* is not inactivated.[10] Hence, *APC* methylation (promoter 1A), though present in younger individuals, does not contribute to gastric carcinogenesis. Methylation of other tumor suppressor and tumor-related genes was present at variable frequencies in non-neoplastic gastric epithelia from elderly individuals (Table 1). There also were differences in methylation frequencies depending on the site in the stomach from which the sample was taken. The exact reasons for these phenomena are unclear. However, the antral location of gastric cancer is known to be susceptible to methylation of several tumor suppressor and tumor-related genes.[11] Intestinal metaplasia, which may be the consequence of *H. pylori*-associated gastritis, especially that of the incomplete type, commonly arises in the antrum and then expands toward the body of the stomach, and may be predisposed to promote methylation of several genes.

*E-cadherin* methylation is apparently age-related in the stomach, and may be accelerated by *H. pylori* infection. However, this methylation also increased with age in the jejunum, ileum, and colon, where *H. pylori*-associated inflammation was unlikely, quite similar to the situation in the stomach.[1] To the best of this author's knowledge and experience, there has been no evidence that there exist differences of susceptibility to age-related methylation between Western and Oriental patients (data not shown). Therefore, more important mechanisms than *H. pylori* infection must still underlie age-related *E-cadherin* methylation.

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## *Class II Transactivator (CIITA) Deficiency in Tumor Cells: Complicated Mechanisms or Not?*

To the Editor-in-Chief:

A severe reduction or absence of expression of major histocompatibility complex (MHC) molecules on tumor cells is frequently noted.[1–3] It is generally accepted that this down-regulation of MHC molecules by tumor cells impairs cellular immune recognition and contributes to inefficient cell-mediated tumor eradication. This low expression or lack of MHC expression is frequently observed in early developmental or embryonically derived tumor cells, and in certain types of hematopoietic cells. These include teratocarcinomas, choriocarcinomas, and neuroendocrine cancers such as neuroblastoma (NB), small cell lung cancer (SCLC), and erythroleukemic cells.[4–11] In addition to a strong reduction or absence of MHC class I expression, these types of tumors are recognized by a lack of interferon-γ (IFN-γ)-mediated induction of MHC class II molecules. This phenotype is generally not found in other tumor types that include melanomas, cervical carcinomas, renal cell carcinomas, head-neck squamous cell carcinomas, gliomas, lymphomas, and tumor virus-transformed normal skin fibroblasts.[6–12]

The MHC is a large multigene family that encodes cell surface glycoproteins involved in binding and presentation of antigenic peptides to T lymphocytes. MHC class I molecules present (tumor) peptides to mainly $CD8^+$ T lymphocytes, whereas the function of MHC class II molecules is to present peptides to $CD4^+$ T lymphocytes. MHC class I molecules are expressed on almost all nucleated cells. In contrast, the constitutive expression of MHC class II molecules is restricted to specific immune cell types that include antigen-presenting cells such as dendritic cells, B-lymphocytes, macrophages, and thymic epithelial cells. Non-immune cells lack constitutive expression of MHC class II, however, in most of these cells MHC class II expression can be induced by IFN-γ.[13,14] Notably, expression of MHC class I molecules can also be enhanced by IFN-γ. Together, the up-regulated expression of MHC class I and class II molecules results in an increase in the immunogenic potential of cells. It is now well established that the class II transactivator (CIITA) plays a pivotal role in the expression of MHC class II and accessory genes (invariant chain and HLA-DM), whereas it has an ancillary function in the expression of MHC class I and β2-microglobulin genes.[15,16] Because both classes of MHC molecules are essential for the generation of antigen-specific immune responses by virtue of their ability to present antigenic peptides to T-lymphocytes, expression and subsequent functional activity of CIITA represents a critical step in the activation of the immune response.

The expression of CIITA in humans is controlled by at least three separate functional promoters, each coding for distinct first exons that drive expression of CIITA during differentiation and in overlapping subsets of different cell types.[17] CIITA promoter I (CIITA-PI) is the promoter used in dendritic cells. Promoter III of CIITA (CIITA-PIII) is constitutively expressed in B-lymphocytes and by activated T-lymphocytes, in monocytes and in dendritic cells. Furthermore, CIITA-PIII can drive CIITA expression after IFN-γ-stimulation in a number of different cell types, including endothelial cells and fibroblasts.[18] While the B-cell-specific expression of CIITA requires a small region directly upstream of the initiation codon, the IFN-γ induction requires an additional region located approximately 5 kb upstream of the transcriptional start site.[18] Promoter IV of CIITA (CIITA-PIV) is the principal IFN-γ inducible promoter.[17–22] Promoter II of CIITA is expressed at insignificant levels and is as yet functionally poorly understood. The cellular and temporal diversity in MHC class II expression is thus regulated via the differential usage of the CIITA promoters.

The first demonstration that lack of IFN-γ-mediated induction of MHC class II antigens was caused by the absence of expression of CIITA was made in fetal trophoblast-derived tumor cell lines.[4,5,9] It was shown that JEG-3 and JAR cells lacked induction of CIITA following exposure of these cells to IFN-γ explaining the absence of expression of all MHC class II isotypes in these tumor cells. Expression of CIITA following gene transfer resulted in the induction and subsequent cell surface expression of all isotypes of MHC class II molecules.[9] Furthermore, in a transfection assay it was shown that CIITA-PIV could be activated to levels similar to those observed in CIITA-inducible cell lines revealing that all transcription factors were present.[6,7,23]

It was subsequently established that the mechanism leading to the lack of IFN-γ-mediated induction of endogenous CIITA was DNA hypermethylation of CIITA-PIII and CIITA-PIV.[6,9,23] This DNA hypermethylation impaired the binding of transcription factors critical to the activation of these promoters resulting in a bare promoter phenotype of CIITA-PIII and CIITA-PIV in trophoblast-derived tumor cells following IFN-γ-induction as established by *in vivo* genomic footprint analysis.[23] Subsequently, we and others also demonstrated that in other types of tumor cells the lack of IFN-γ-mediated induction of CIITA was caused by promoter hypermethylation both in humans[6] and in mice.[24] We noted that this phenomenon was found predominantly in human developmental tumor cell lines.[6] These developmental tumor cell types included neuroblastomas and teratocarcinomas. Treatment of these cell lines with t-azacytidine resulted in the restoration of both CIITA-PIII and CIITA-PIV promoter activity and resulting expression of CIITA congruent with induction of MHC class II genes.[6] We have argued that silencing of CIITA through promoter