

Mandatory fortification with folic acid in the United States appears to have adverse effects on histone methylation in women with pre-cancer but not in women free of pre-cancer

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Objective: To evaluate whether mandatory fortification of grain products with folic acid in the US is associated with changes in histone methylation in cells involved in cervical carcinogenesis.

Methods: Cervical specimens obtained before (1990 to 1992) and after mandatory folic acid fortification (2000 to 2002) were used to examine the degree of histone methylation (H3 Lys-9) by immunohistochemistry. 91 women (51 before and 40 after fortification) were diagnosed with cervical intraepithelial neoplasia (CIN) grade 3 or carcinoma in situ (CIS) and sections utilized in the study also contained normal, reactive or metaplastic cervical epithelium, CIN 1 or CIN 2. 64 women (34 before and 30 after fortification) were free of CIN and these sections contained only normal or reactive cervical epithelium. Immunohistochemical staining for H3 Lys-9, its assessment in different cell or lesion types and data entry were blinded for fortification status. For each cell type or lesion category we used PROC MIXED in SAS with the specimen identifier as a random effect and the robust variance estimator to estimate age- and race-adjusted intensity score for H3 Lys-9 in the pre- and post-fortification periods.

Results: Degree of H3 Lys-9 methylation was significantly higher ($P < 0.0001$) in \geq CIN 2 lesions (CIN 2, CIN 3 and CIS) than in \leq CIN 1 lesions (CIN 1, normal, reactive and metaplastic), in both pre- and post-fortification CIN 3/CIS specimens. Age- and race-adjusted mean H3 Lys-9 score was significantly higher in all cell or lesion types in CIN 3/CIS specimens obtained in the post-fortification period compared to pre-fortification period ($P < 0.05$, all comparisons). In contrast, in specimens obtained from women free of CIN, Lys-9 methylation in normal/reactive cervical epithelium was significantly lower in post-fortification specimens than in pre-fortification specimens ($P = 0.03$).

Conclusions: Higher levels of Lys-9 methylation in \geq CIN 2 compared to \leq CIN 1 lesions suggest that higher Lys-9 methylation is associated with progression of lower grade CIN to higher grade CIN. Higher Lys-9 methylation in cervical tissues of women diagnosed with CIN 3 in the post-fortification period than in pre-fortification period suggest that fortification may adversely affect histone methylation in already initiated cells. Lower Lys-9 methylation in normal/reactive cervical cells of women free of CIN in the post-fortification period than pre-fortification on the other hand suggests that fortification is likely to protect against initiation of carcinogenic process in the cervix. These results suggest that mandatory fortification with folic acid in the US seems to have different effects on cancer depending on the stage of carcinogenesis. Because this is the first study to report folic acid fortification-associated differences in histone methylation and because of the limitations inherent to the approach we have taken to demonstrate these differences, validation of the results in other study populations or with other techniques for assessing histone methylation is necessary.

Keywords: folic acid, fortification, histone methylation, cervix

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Background

The addition of folic acid (synthetic form of folate, a water-soluble B vitamin) to grain products which was mandated by the Food and Drug Administration (FDA) in 1998 was directed towards the prevention of neural tube defects (NTDs). The potential effects of increasing folate intake population-wide on other human health conditions are largely unclear at this point, but concerns have been raised about possible adverse effects of higher folate status on cognitive impairment and anemia,¹ immune function,² reducing the efficacy of anti-epileptic drugs or anti-folate chemotherapy or promoting the progression of initiated cancer cells. Since folic acid fortification began in the USA, supra-physiologic serum folate concentrations (>45 nmol/L or >19.8 ng/mL) have been documented in about 25% of the US population.³ These supra-physiologic folate concentrations are likely to be associated with the presence of unmetabolized folic acid.⁴ Evidence suggests that unmetabolized folic acid found in blood after the ingestion of supplements or fortified foods may have adverse effects on folate binding proteins or transporters⁵ possibly interfering with normal folate metabolism. Although it is possible that unmetabolized folic acid may have different effects on already initiated cancer cells compared to uninitiated cells, this has not been shown directly for any cancer type. However, it is also important to understand that fortification will not only raise the concentrations of unmetabolized folic acid but also the concentrations of total folate in the body which could have disease protective effects.

Because the changes in total folate status and/or the presence of un-metabolized folic acid as a result of folic acid fortification are likely to alter folate-related molecular mechanisms which are hallmarks of cancer, such as the methylation of DNA and histones, or the expression of folate pathway enzymes such as DNA methyl transferases (Dnmts), we have investigated changes in such events in relation to the folic acid fortification program in the USA. We have previously reported that global DNA methylation (assessed by an immunohistochemical method) was not significantly different in cervical tissues of women diagnosed with cervical intraepithelial neoplasia (CIN 3), in the pre-folic acid fortification period compared to post-folic acid fortification period.⁶

Our recent results also suggested that degree of expression of DNA methyltransferase 1 (Dnmt 1) was significantly higher in all lesion types detected in women diagnosed with CIN 3 in the post-fortification period compared to pre-fortification period.⁷ Because the degree of Dnmt 1 expression was significantly higher in \geq CIN 2

lesions compared to \leq CIN 1 lesions, we believe that higher expression of Dnmt 1 may be a risk factor for the development of cervical cancer (CC). Therefore, folate fortification is likely to exert adverse effects in women diagnosed with pre-cancer lesions possibly via alterations in Dnmt 1.

Importantly, however, expression of Dnmt 1 assessed by the same method in cervical epithelium of women free of CIN in the two fortification periods ($n = 34$, pre and $n = 30$, post) demonstrated that the degree of Dnmt 1 expression was not significantly different between the two fortification periods (unpublished data). Although these women are free of CIN, they are at high risk for developing CC because they were referred for evaluation by colposcopically directed biopsies as a result of abnormal pap diagnoses and therefore likely to be exposed to carcinogenic types of human papillomaviruses (HPVs), the main risk factor for CC. These observations and results suggested that higher folate intake due to folic acid fortification and its possible effects on the expression of Dnmt 1 expression are likely to increase the risk of malignant transformation of initiated cells as in CIN 3 to CC, but these fortification associated changes may not pose a risk for those in women who are at risk for CC, but not diagnosed with cervical pre-cancer (uninitiated cells). This is an interesting and possible differential effect of higher folic acid on initiated cells compared to uninitiated cells. The purpose of the current study was to investigate whether folic acid fortification has similar differential effects on histone methylation, another hallmark event for cancer.

Materials and methods

Study design

The study population consisted of women at risk for developing CC based on abnormal pap diagnoses reported by Health Departments in Alabama who were referred to a colposcopy clinic at the University of Alabama at Birmingham (UAB) for further examination by colposcopically directed cervical biopsies. Paraffin-embedded biopsy specimens, archived in the Department of Pathology at the University of Alabama at Birmingham (UAB) from these women were used to evaluate the degree of H3 Lys-9 histone methylation in women diagnosed with CIN 3 lesions where precancerous cells are in the entire epithelial layer of the cervix or in women free of CIN before (1990 to 1992) and after the initiation of the national program of fortification of grains with folic acid (2000 to 2002). We used randomly selected specimens from 51 and 40 women diagnosed with CIN 3 (based on available pathology report) from the pre- and post-fortification periods, respectively, and specimens from 34 and 30 women free of

CIN (based on available pathology report) from the pre- and post-fortification periods respectively. Tissue sections stained with H and E were requested for all these specimens and the study pathologist (WCB) reviewed them to identify CIN 3 lesions or other lesions in the same specimen (normal, reactive or metaplastic cervical epithelium, CIN 1 [only the lower one-third of cells in the epithelial layer of the cervix are abnormal], CIN 2 [up to two-thirds of the epithelial layer contains precancerous cells] or CIS [precancerous cells are in the entire epithelial layer of the cervix]). Sections from women free of CIN were also examined by WCB to assure that they were free of CIN lesions. We reviewed medical charts in an attempt to obtain additional data on, for example, smoking and use of vitamin supplements, but this information was unavailable for most women and therefore was not used in this study. Data on race and age were available for all women. All specimens were given a code and the individuals who were involved with immunohistochemical staining, evaluation of H3 Lys-9 histone methylation and data entry were blinded for the fortification status.

Histological criteria and classification of lesions

Seven histological cell or lesion types, namely, normal, reactive, metaplastic cervical epithelial cells, CIN 1, CIN 2, CIN 3 or CIS were identified by the study pathologist in specimens specified as CIN 3 by the pathology report (51 women in the pre-fortification period and 40 women in the post-fortification period). All these cell or lesion types found in each section were evaluated separately for H3 Lys-9 histone methylation. The total number of lesions identified in pre-fortification specimens was 112 (34 normal, 18 reactive, 2 metaplastic, 5 CIN 1, 6 CIN 2, 38 CIN 3 and 9 CIS). The total number of lesions identified in post-fortification specimens was 98 (34 normal, 23 reactive, 2 metaplastic, 1 CIN 2, 28 CIN 3 and 10 CIS). All specimens specified as free of CIN by the pathology report (34 in the pre-fortification period and 30 in the post-fortification period) contained normal or reactive cervical epithelium only.

Immunohistochemical analysis

Our methods of immunohistochemical analysis with various antibodies (with/without various antigen retrieval techniques) have been reported previously.^{8,9} A comparison of results with and without different antigen retrieval techniques revealed that the protocol described below gave the best results for the polyclonal antibody specific for anti-dimethyl-histone 3 (Lys9, Upstate 07-212). Briefly, the slides containing 4 µm

thick paraffin tissue sections were deparaffinized in xylene, placed in 0.01 M citric acid, pH 6.0, and boiled in a pressure cooker set at full power for 10 minutes. After antigen retrieval, the sections were cooled and rinsed in deionized H₂O (dH₂O). The sections were then rinsed with Tris-buffered saline and treated with 3.0% H₂O₂ for 5 minutes to quench endogenous peroxidase activity. Sections were incubated with preimmune goat serum (3%) for 20 minutes at room temperature to suppress nonspecific staining and then subsequently incubated with Lys9 antibody (1:50 concentration) for 1 hour at room temperature. After washing thoroughly with Tris-buffered saline, the primary antibody was detected using a multi-species system (Signet Laboratories, Inc., Dedham, MA, USA). The antibody-antigen complex was visualized using a 3, 3'-diaminobenzidine substrate kit according to the manufacturer's instructions (Biogenex, Inc., San Ramon, CA, USA); and lightly counterstained with haematoxylin. Slides were sequentially dipped in 70%, 95% and 100% ethanol (3 minutes each); and then were allowed to dry slightly. Finally, slides were dipped in xylene 3 times (3 minutes each) after which they were protected with cover slips.

Assessment of immunostaining

Immunostaining for H3 Lys-9 was localized mainly in the nuclei of cells. Nuclear staining in cells with or without cytoplasmic staining was interpreted as a positive reaction. The degree of H3 Lys-9 methylation in the nuclei was recorded independently by two observers (WCB and JEC) as a percentage of cells positive for H3 Lys-9 based on a visual assessment of the intensity of brown reaction product within the cell nucleus on a scale of 0 (no staining) to 4+ (intense staining). All evaluations were done separately in the basal, bottom-half and top-half layer of the normal, reactive and metaplastic cervical epithelium and CIN 1, 2 and 3. This approach is time consuming, but allows obtaining a more accurate estimate on the percentage of cells positive for H3 Lys-9 in each cell or lesion type. Because the basal, bottom-half and top-half layers cannot be separated in CIS, one reading was given for those lesions. The degree of H3 Lys-9 methylation was reported as an intensity score which was derived by multiplying the percentage of cells positive for H3 Lys-9 at each intensity score by the appropriate intensity score. For all cell or lesion types the intensity score in the basal, bottom-half and top-half layer were averaged to obtain the average degree of H3 Lys-9 methylation in each cell or lesion type. The readings from the two observers were averaged for each cell or lesion type and thus the degree of

H3 Lys-9 methylation reported is the average of the two observers.

Statistical analysis

In the analyses, the dependent variable was the staining intensity score of H3 Lys-9. The independent variables of interest were 1) the period of diagnosis (before or after implementation of the folate fortification program) and 2) in CIN 3 specimens, the diagnostic category of the tissue examined combined into \leq CIN I which included normal, reactive or metaplastic cervical epithelium and CIN 1 or \geq CIN 2 which included CIN 2, CIN 3 and CIS. This grouping is based on the clinical significance of the pathological findings: the \leq CIN I group included tissue with findings that are not considered to be true pre-neoplastic, whereas the \geq CIN 2 category included CIN 2, CIN 3/CIS, which are considered to be true pre-neoplastic lesions of the cervix. Because a patient's specimen could contain areas with pathologic findings compatible with more than one category, the degree of H3 Lys-9 methylation was evaluated independently in each area and the same woman may have contributed information on the degree of H3 Lys-9 methylation in more than one diagnostic category. We used mixed linear models and robust variance estimators to properly take into account the potential correlation of multiple findings from the same subject. The independent variables of interest in women free of CIN were 1) the period of diagnosis (before or after implementation of the folic acid fortification program) 2) normal or reactive cervical epithelium combined, as there was no difference in staining intensities between these two categories.

For each cell type or lesion category we used PROC MIXED in SAS with the specimen identifier as a random effect and the robust variance estimator to estimate age- and race-adjusted intensity score for H3 Lys-9 in the pre- and post-fortification periods. The differences in intensity scores in the pre-fortification compared to the post-fortification group were considered significant at $P < 0.05$. To determine whether the pattern of H3 Lys-9 methylation has changed with fortification in women diagnosed with CIN 3, we assessed whether the intensity score for H3 Lys-9 in \leq CIN 1 and \geq CIN 2 specimens in the pre-fortification period was similar to that in the post-fortification period. In an additional analysis we considered the within-woman variation in the degree of H3 Lys-9 methylation across diagnostic categories. To accomplish this, we summed the H3 Lys-9 positive cells in all \leq CIN 1 lesions and subtracted the total from the sum for H3 Lys-9 positive cells in \geq CIN 2 lesions, to obtain a within-woman H3 Lys-9 difference. Next, we compared the

distribution of within-woman H3 Lys-9 differences in the pre- and post-fortification period. The mean difference in the pre-fortification period was compared to that from the post-fortification using a t-test.

Results

As shown in Table 1, in the pre-fortification period, 67% of the women diagnosed with CIN 3 were Caucasian Americans (CAs) and the rest were African-Americans (AAs) while in the post-fortification group 63% were CAs and the rest AAs. The mean age of the pre-fortification group (33.73 ± 13.12) was similar to that of the post-fortification group (31.55 ± 8.71). Among women free of CIN, in the pre-fortification period, 44% were Caucasian Americans (CAs) and the rest were African-Americans (AAs) while in the post-fortification group 70% were CAs and the rest AAs and the mean age of the pre-fortification group (25.15 ± 4.91) was younger than the post-fortification group (41.90 ± 10.65).

Age- and race-adjusted H3 Lys-9 score was significantly higher in all lesion types detected in CIN 3 specimens from the post-fortification period compared to the pre-fortification period ($P < 0.05$, all comparisons). Similarly, when lesions were combined into two main groups (\leq CIN 1 lesions and \geq CIN 2 lesions), the H3 Lys-9 score were significantly higher in both \leq CIN 1 lesions and \geq CIN 2 lesions in the post-fortification period compared to the pre-fortification period ($P < 0.005$ for all comparisons). The degree of H3 Lys-9 methylation was significantly higher in \geq CIN 2 lesions compared to \leq CIN 1 lesions in both pre- and post-fortification CIN3 specimens. The age- and race-adjusted H3 Lys-9 score was significantly lower in normal or reactive cervical epithelium in specimens free of CIN in the post-fortification period compared to the pre-fortification period ($P = 0.0262$).

Figure 1 demonstrates the H3 Lys-9 methylation status of CIN 3 and normal cervical epithelium detected in the same section; higher degree of H3 Lys-9 methylation in a CIN 3 lesion (B) compared to normal cervical epithelium (A) in pre-fortification period, higher degree of H3 Lys-9 methylation in a CIN 3 lesion (D) compared to normal cervical epithelium (C) in post-fortification period and higher degree of H3 Lys-9 methylation in normal cervical epithelium in the post-fortification period (C) compared to pre-fortification period (A) and higher degree of H3 Lys-9 methylation in CIN 3 in the post-fortification period (D) compared to pre-fortification period (B). Figure 2 demonstrates the lower H3 Lys-9 methylation in normal cervical epithelium in the

Table I Degree of expression of H3 Lys-9 in cervical cells in the pre- and post-fortification periods

Specimens of CIN 3	Pre-fortification (1990–1992)			Post-fortification (2000–2002)			P values ^b
	AA	CA	AA + CA	AA	CA	AA + CA	
Number of subjects	17	34	51	15	25	40	
Age, years (mean ± SD)	33.00 ± 13.35	34.09 ± 13.19	33.73 ± 13.12	30.07 ± 7.29	32.44 ± 9.49	31.55 ± 8.71	
Intensity score for H3 Lys-9 ^a							
≤CIN 1	0.46 ± 0.10	0.57 ± 0.07	0.51 ± 0.06	0.73 ± 0.10	0.85 ± 0.06	0.79 ± 0.06	0.0024
≥CIN 2	0.71 ± 0.10	0.80 ± 0.10	0.76 ± 0.07	1.15 ± 0.12	1.17 ± 0.07	1.16 ± 0.07	0.0002
Specimens free of CIN							
Number of subjects	19	15	34	9	21	30	
Age, years (Mean ± SD)	25.21 ± 5.75	25.07 ± 3.77	25.15 ± 4.91	46.00 ± 14.71	40.14 ± 8.19	41.90 ± 10.65	
Intensity score for H3 Lys-9 ^a							
Normal or reactive cervical epithelium	0.71 ± 0.07	0.70 ± 0.10	0.71 ± 0.06	0.54 ± 0.06	0.52 ± 0.05	0.53 ± 0.04	0.0262

^aValues are means ± SE, adjusted for age and race; ^bP comparing AA + CA for pre- and post-fortification.

Abbreviations: AA, African Americans; CA, Caucasian Americans; CIN, cervical intraepithelial neoplasia.

post-fortification period (B) compared to pre-fortification period (A) in specimens free of CIN.

Discussion

Histones are a group of evolutionarily conserved proteins that package and organize DNA in cells. Chemical modification of histones such as methylation results in aberrant gene regulation, DNA damage, cell cycle defects, genomic instability, all of which are hallmarks of cancer.¹⁰ Recently, altered histone methylation has also emerged as key to DNA methylation-related gene silencing and this type of gene silencing in cancer is accompanied by an increase in H3 Lys9 methylation.¹¹ We observed that the degree of H3 Lys-9 methylation is higher in higher grade lesions (≥CIN 2) compared to lower grade lesions (≤CIN 1). This suggests that increases in H3 Lys9 methylation are likely to facilitate malignant transformation of cervical cells at risk to pre-cancerous cells or cancerous cells. Our results suggest that the degree of H3 Lys9 methylation is higher in women diagnosed with CIN 3 in the post-folic acid fortification period compared to pre-folic acid fortification period. Because CIN 3 is a precursor lesion for the development of invasive CC and because the transformation of CIN 3 to invasive CC is likely to be accelerated by higher histone methylation, these observations raise concerns about the possibility that mandatory folic acid fortification program in US may have hastened progression to cancer in women diagnosed with pre-cancer. On the other hand, higher folate status in the post folic acid era is associated with lower histone methylation in women at risk for developing cervical pre-cancer, but free of precancerous

changes and this is likely to prevent transformation of HPV exposed cervical epithelium to pre-cancer or cancer.

Mechanisms by which folate may alter histone methylation are not clearly understood. However, a typical western diet characterized by lower intakes of folate-rich natural foods has been shown to lower the ratio of S-adenosyl-methionine (SAM) versus S-adenosyl-homocysteine (SAH), and a low SAM/SAH ratio is known to inhibit the activity of several methyltransferases.¹² SAM/SAH ratio is likely to be higher in the post fortification era due to higher methyl availability and conversion of SAH to SAM and this may result in higher expression of histone methyltransferases and thus higher histone methylation in initiated cells. Because we observe significantly opposite effects of higher folate on histone methylation in uninitiated cells, altered SAH/SAM is likely to have different effects on methyltransferases based on the stage of the carcinogenic process.

The differential effects of folic acid fortification on histone methylation in initiated versus uninitiated cells may also be explained by the influence of Dnmt 1 on histone methylation. Treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5Aza-dC) is shown to reduce Lys-9 methylation at silenced loci and results in reactivation of tumor suppressor genes¹³ suggesting that the observed higher Dnmt expression in CIN 3 lesions may have contributed to higher histone methylation in CIN 3 lesions. Expression of Dnmt 1 was not higher in the post-folic acid period in uninitiated cells and this may explain the lower histone methylation in normal/reactive cervical epithelium in the post-folic acid period. Although further research is

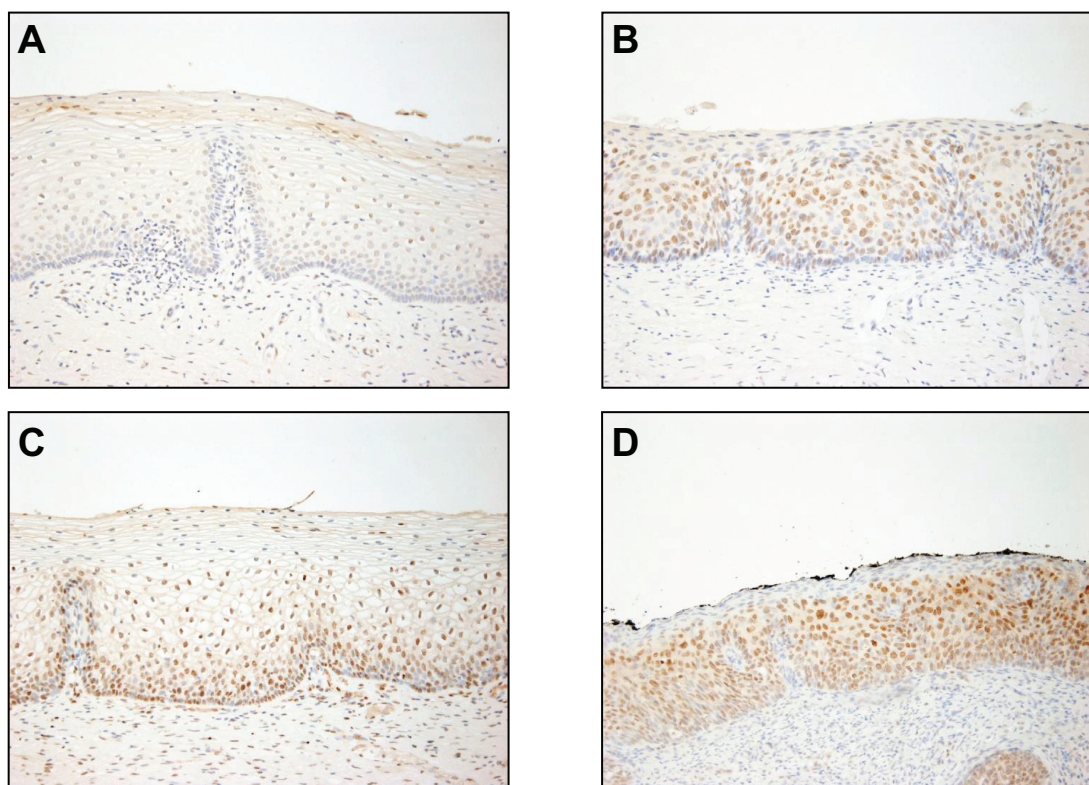


Figure 1 Expression of H3 Lys-9 methylation in pre-fortification normal cervical epithelium (**A**); pre-fortification CIN 3 lesion (**B**); post-fortification normal cervical epithelium (**C**) and post-fortification CIN 3 lesion (**D**).

necessary to clarify this relation, the explanation is biologically plausible because previous research has established a direct connection between the enzymes responsible for DNA methylation and histone methylation.¹⁴

These preliminary findings should be interpreted in light of the limitations inherent to a study of this nature. For example, pre- and post-fortification specimens were collected several years apart and therefore, we cannot completely

exclude the possibility that observed differences in degree of H3 Lys-9 methylation are an effect of storage time on antibody binding, or due to time-dependent changes in the quality of the DNA. However, because we demonstrate either no change or lower degree of expression in Dnmt 1 or H3 Lys-9, respectively in post-fortification compared to pre-fortification suggest that time-dependent changes in DNA are unlikely to explain the observed differences in degree

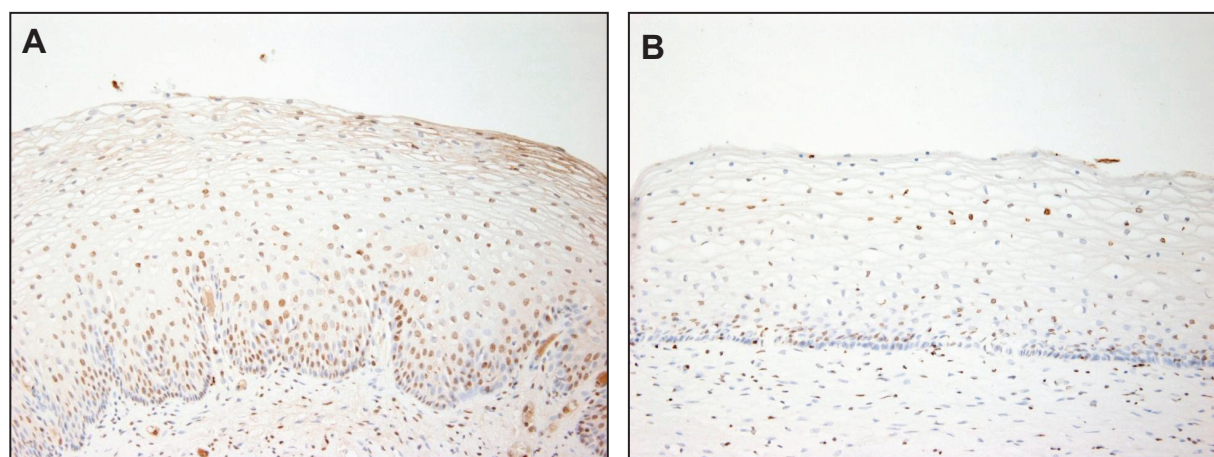


Figure 2 Expression of H3 Lys-9 methylation in pre-fortification normal cervical epithelium (**A**) and post-fortification normal cervical epithelium (**B**) in a specimen free of CIN.

of H3 Lys-9 methylation. Because of the cross-sectional design, this study cannot demonstrate a causal link between altered H3 Lys-9 methylation and cancer risk. We attempted to associate degree of H3 Lys-9 methylation with disease risk by evaluating recurrence of pre-cancer, progression to invasive cancer or survival of these patients, but were unable to retrieve this information from patient records. For other cancers (example, colorectal), histone methylation is shown to be a critical modification responsible for maintenance of DNA methylation-related gene silencing, an important risk profile for cancer development and prognosis. Therefore, the observed association between folic acid fortification and changes in histone methylation in cervical tissues are important preliminary findings.

Another limitation of our study is that at the individual subject level, we have no environmental or lifestyle data and data to support that folic acid intake, blood levels or cellular levels of folate have increased as a result of fortification. In a population-based study, however, it is fair to assume that everyone had the opportunity to be exposed to fortified food items after fortification had begun and population-based studies have shown that serum folate concentrations have significantly increased after folic acid fortification.¹⁵ Although the limitations cited call for caution in interpreting the results generated by this study, the finding that the response of initiated cells to higher folic acid may be different from that of normal or uninitiated cells¹⁶ is biologically plausible and warrants further investigation.

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