Published in final edited form as:
Nutrition. 2008 January ; 24(1): 94–99.

Mandatory Fortification with Folic Acid in the United States is associated with Increased Expression of DNA Methyltransferase 1 in the Cervix

Chandrika J Piyathilake[1], **Jorge E Celedonio**[1], **Maurizio Macaluso**[2], **Walter C Bell**[3], **Maria Azrad**[1], and **William E Grizzle**[3]

1*Department of Nutrition Sciences, The University of Alabama at Birmingham, AL 35294*

2*School of Public Health, The University of Alabama at Birmingham, AL 35294*

3*Department of Pathology, The University of Alabama at Birmingham, AL 35294*

Abstract

Objective—The objective of this study was to evaluate whether mandatory fortification of grain products with folic acid in the USA is associated with changes in DNA methyltransferase (Dnmt) 1 expression in cells involved in cervical carcinogenesis.

Methods—Archived specimens of cervical intraepithelial neoplasia (CIN) diagnosed before (1990-92) and after mandatory folic acid fortification (2000-02) were used to examine the expression of Dnmt 1 in specific lesions involved in cervical carcinogenesis by immunohistochemistry. The total number of lesions examined was 101 in the pre-fortification period and 96 in the post-fortification period. Immunohistochemical staining for Dnmt 1, its assessment and data entry were blinded with regard to the fortification status.

Results—Age- and race-adjusted mean percentage of cells positive for Dnmt 1 or the Dnmt 1 score was significantly higher in all lesion types (i.e., normal cervical epithelium, reactive cervical epithelium, metaplastic cervical epithelium, CIN or carcinoma in situ) detected in the post-fortification period compared to pre-fortification period ($P < 0.05$, all comparisons). The degree of Dnmt 1 was significantly higher ($P < 0.0001$) in ≥ CIN 2 lesions compared to ≤ CIN 1 lesions, regardless of the fortification group.

Conclusions—These results suggest that mandatory fortification with folic acid in the United States seem to have resulted in a change in the degree of expression of Dnmt 1 in cells involved in cervical carcinogenesis. Because the approach we have taken to demonstrate these differences have limitations inherent to a study of this nature and this is the first study to report a folate fortification associated change in Dnmt 1, validating these results in other study populations and or with other techniques of assessing Dnmt 1 will increase the scientific credibility of these findings.

Keywords

Folic acid; Fortification; DNA Methyltransferase; Cervix

Correspondence to: Chandrika J Piyathilake, Department of Nutrition Sciences, Division of Nutritional Biochemistry and Genomics, University of Alabama at Birmingham (UAB), University Station, Birmingham, AL 35294. Phone: 205-975-5398, Fax: 205-996-2859, Email: piyathic@uab.edu.

Potential conflicts of interest: None

Background

Although 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 toward the prevention of neural tube defects (NTDs), several other disease risks including cancer may be affected by this. Because the methylation status of DNA plays an important role in modifying cancer risk, we recently evaluated the status of global DNA methylation in relation to the fortification program. We observed that the degree or the pattern of global DNA methylation in tissues of the cervix is not altered by the fortification (1). A recently described mathematical simulation of folate-mediated 1-carbon metabolism (2) reported that intracellular methionine concentrations are relatively insensitive to variation in total folate, but intracellular concentration of S-adenosyl-methionine (SAM) had a strong linear association with total folate concentration suggesting that SAM concentrations are likely to be elevated due to higher folate intake as a result of the folic acid fortification. Even a short term supplementation with folic acid (1.2 mg/day for 12 week) has been shown to increase whole blood SAM in humans (3). Because SAM is the principal substrate for cellular methylation reactions, it is reasonable to expect that the overall methylation rate would be a function of SAM concentration and, therefore, the total folate pool. However, these recent simulations suggested instead that allosteric interactions between the folate and methionine cycles stabilize the Dnmt reaction rate against variations in methionine input and folate pool size. This phenomenon may explain the no change in global DNA methylation after higher folate intake after the fortification program. However, it is also known that Dnmt is inhibited by *S*-adenosylhomocysteine (SAH) and SAH is likely to be lower in the post fortification era due to higher methyl availability for its conversion to SAM. This may result in higher expression of Dnmt. Inhibition of Dnmt via high SAH has been shown to reduce methylation in promoter regions of genes, but not at the level of global DNA methylation (4). Whether the opposite effect may hold true, namely, reduced inhibition of Dnmt because of lower SAH is not reported, but this possibility cannot be excluded. If this does occur, folate fortification may be associated with higher expression of Dnmt 1 and this may result in higher methylation in the promoter regions of specific genes without significant effects on global DNA methylation. In this study, we investigated whether the Dnmt is over expressed in cervical tissues obtained in post fortification period (2000-2002) compared to its expression in similar tissues obtained prior to the fortification (1990-92).

Materials and methods

Study design

Paraffin-embedded specimens, archived in the Department of Pathology at the University of Alabama at Birmingham (UAB), were used to evaluate the degree of expression of Dnmt 1 in women diagnosed with CIN lesions before (1990-92) and after the initiation of the national program on fortification of grains with folic acid (2000-02). The specimens were selected as follows. First, we obtained diagnostic retrieval reports (which contain the date of diagnosis, accession number, type of specimen [biopsy, curettage, etc], adequacy of specimen and diagnosis) of women diagnosed with CIN in the two periods of interest, 1990-92 and 2000-02. We prepared a list of all available specimens from this initial screening for women diagnosed with different degrees of CIN (CIN 1, 2 and 3) according to the pathology report. In this manuscript we used the first 46 and 38 subjects diagnosed with CIN 3 (according to the UAB pathology report) from the pre- and post-fortification periods respectively. The sections stained with H and E were requested for all these specimens and the study pathologist (WB) reviewed them to identify the different types of epithelial cells or lesions (normal, reactive or metaplastic cervical epithelium, CIN 1, CIN 2, CIN 3 or carcinoma *in situ* [CIS]). We reviewed medical

charts in an attempt to obtain additional data on smoking, use of vitamin supplements, etc 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 the degree of Dnmt 1 expression and data entry were blinded with regard to 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 in this study based on the histological features of cervical epithelium in cervical sections obtained from women who were diagnosed with CIN 3 or CIS (46 women in the pre-fortification period and 38 women in the post fortification period). All cell or lesion types found in each section were evaluated separately for the expression of Dnmt 1. The total number of lesions identified in pre-fortification specimens was 101 (33 normal, 10 reactive, 5 metaplastic, 4 CIN 1, 11 CIN 2, 24 CIN 3 and 14 CIS). The total number of lesions identified in post-fortification specimens was 96 (32 normal, 14 reactive, 3 metaplastic, 4 CIN 1, 1 CIN 2, 27 CIN 3 and 15 CIS).

Immunohistochemical Analysis

Our methods of immunohistochemical analysis with various antibodies (with/without various antigen retrieval techniques) have been reported previously [5,6]. 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 Dnmt 1 (H-300, sc-20701). Briefly, the slides containing 4μ thick paraffin tissue sections were deparaffinized in xylene, placed in 0.01M 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, rinsed in deionized H_2O (dH_2O), and placed in 3.5 N HCl for 15 minutes at room temperature to open the DNA. The sections were rinsed with dH_2O and treated with 3.0% H_2O_2 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 Dnmt 1 antibody (1:100 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). 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 three times (3 minutes each) after which they were protected with cover slips.

Assessment of Immunostaining

Immunostaining for Dnmt 1 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 expression of Dnmt 1 in the nuclei was recorded independently by two observers (WCB and JEC) as a percentage of cells positive for Dnmt 1 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 Dnmt 1 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 expression of Dnmt 1 was reported as the total percentage of cells positive at any intensity (1-4 +) and as an intensity score which was derived by multiplying the percentage of cells positive

for Dnmt 1 at each intensity score by the appropriate intensity score. For all cell or lesion types except for CIS, the percentage of cells positive for Dnmt 1 or the intensity score in the basal, bottom-half and top-half layer were averaged to obtain the average degree of Dnmt 1 expression 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 Dnmt 1 expression reported is the average of the two observers. In order to assure the reproducibility of results, we re-evaluated staining patterns in randomly selected 20 cases each from the pre and post-fortification periods.

Statistical analysis

In the analyses the dependent variable was either the percentage of cells positive for Dnmt 1 or the Dnmt 1 staining intensity score. The independent variables of interest were 1) the period of diagnosis (before or after implementation of the folate fortification program) and 2) the diagnostic category of the tissue examined combined into ≤ CIN I which included normal, reactive or metaplastic cervical epithelium and CIN 1 or ≥ CIN 2 which included CIN 2, CIN 3 and CIS. This grouping is based on the clinical significance of the pathological findings: the ≤ CIN I group included tissue with findings that are not considered to be true pre-neoplastic, whereas the ≥ CIN 2 category included CIN 2, CIN 3 and 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 expression of Dnmt 1 was evaluated independently in each area and the same woman may have contributed information on the degree of Dnmt 1 expression in more than one diagnostic category. We used statistical methods that appropriately take into account the potential correlation of multiple findings from the same subject.

For each diagnostic category we used the mixed procedure method in SAS with specimen ID as a random effect and the robust variance estimator to estimate age- and race-adjusted mean percentages of cells positive for Dnmt 1 or the intensity score for Dnmt 1 in the pre- and post-fortification period. The difference in mean percentage of cells or intensity score in the pre-fortification compared to the post-fortification group were considered significant at $p < 0.05$. To determine whether the pattern of Dnmt expression has changed with fortification, we assessed whether the percentage of cells positive for Dnmt 1 or the intensity score for Dnmt 1 in ≤ CIN 1 and ≥ 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 Dnmt 1 expression across diagnostic categories. To accomplish this, we summed the Dnmt 1 positive cells in all ≤ CIN 1 lesions and subtracted the total from the sum for Dnmt 1 positive cells in ≥ CIN 2 lesions, to obtain a within-woman Dnmt 1 difference. Next, we compared the distribution of within-woman Dnmt 1 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. A similar approach was taken to compare the results from the re-evaluated 20 cases each from the pre and post-fortification periods.

Results

As shown in Table 1, in the pre-fortification period, 79% of the women were Caucasian Americans (CAs) and the rest were African-Americans (AAs) while in the post-fortification group 72% were CAs and the rest AAs. The mean age of the pre-fortification group (34.3 ± 13.5) was similar to that of the post-fortification group (31.7 ± 8.8). The expression of Dnmt 1, % cells positive or score were significantly higher in both ≤ CIN 1 lesions and ≥ CIN 2 lesions in the post-fortification period compared to the pre-fortification period ($p < 0.0001$ for all comparisons). The expression of Dnmt 1 was significantly higher in ≥ CIN 2 lesions compared to ≤ CIN 1 lesions in both pre and post fortification samples. Results from the re-evaluated 20 cases each from the pre and post-fortification periods demonstrated exactly the

same staining patterns (data not shown in the manuscript, but was presented to the reviewers). Figure 1 demonstrates the higher expression of Dnmt 1 in a CIN 3 lesion (B) compared to normal cervical epithelium (A) in pre-fortification period, higher expression of Dnmt 1 in a CIN 3 lesion (D) compared to normal cervical epithelium (C) in post-fortification period and higher expression of Dnmt 1 in normal cervical epithelium in the post-fortification period (C) compared to pre-fortification period (A) and higher expression of Dnmt 1 in CIN 3 in the post-fortification period (D) compared to pre-fortification period (B).

Discussion

Dnmt catalyzes the transfer of a methyl group from S-adenosylmethionine (AdoMet) to the 5-position of cytosine in DNA and the specific pattern of methylation provides differential accessibility to the DNA information code by affecting DNA-protein interactions and chromatin condensation (7). Dnmt 1 has been shown to recognize and binds with high affinity to DNA lesions such as base mispairs, uracil, and other unusual DNA conformations (8,9) and these lesions are thought to be present in human pre-neoplastic lesions. This phenomenon may explain the higher expression of Dnmt 1 in ≥ CIN 2 lesions compared to ≤ CIN 1 lesions. It has been hypothesized that the high-affinity binding of the Dnmt 1 to un-repaired lesions in DNA could sequester available enzyme away from the replication fork and promote passive replication-dependent demethylation (10). It has also been hypothesized that when DNA lesions occur within the promoter region of cancer-promoting genes the aberrant high-affinity binding of the methyltransferase to these lesions is associated with ectopic cytosine methylation. Therefore, in general, the increased Dnmt 1 activity is associated with genome-wide hypomethylation and regional hypermethylation in CpG islands of tumor suppressor genes (11) and both of these DNA changes are associated with increased risk of cancer. Therefore, higher Dnmt 1 expression in the post fortification era is likely to have significant risk modification effects on cancer. However, the degree of over-expression of Dnmt 1 required for these changes to occur is unknown and may vary by the individual or population level or may depend on specific tissues. In our recently published manuscript from the same study, we observed that genome-wide methylation of DNA has not changed after fortification suggesting that the degree of over-exprerssion of Dnmt 1 seen in this population is unlikely to be associated with higher risk of cancer due to global DNA hypomethylation. However, because there have been concerns about potential harmful effects of high folate intake on cancer risk (12,13), and the association between promoter methylation and folate status is controversial (some studies demonstrating that the prevalence of promoter hypermethylation was higher in individuals with low folate intake (14) while others demonstrating an association between high folate levels and frequent DNA hypermethylation [15]) and these concerns are still not answered, the post fortification changes in Dnmt expression, global and gene-specific methylation should be carefully evaluated in relation to cancer risk in future studies.

Although we demonstrate clearly that Dnmt 1 expression is higher in post-fortification samples, the approach we have taken to demonstrate these differences have limitations inherent to a study of this nature. Because the pre and post samples have been collected several years apart, we cannot completely exclude the possibility that observed differences in Dnmt 1 expression is an effect of storage time on antibody binding or due to time-dependent changes in quality of DNA. However, other markers evaluated by immunohistochemistry in this study (global methylation, published [1] and histone methylation and DNA damage/stability markers, unpublished at this point) demonstrate either no change or lower degree of expression in post fortification compared to pre-fortification suggest that time-dependent changes in DNA is unlikely to explain the observed results for Dnmt 1. Because of the cross-sectional study design, this study is unable to demonstrate a link between Dnmt 1 expression and cancer risk. We attempted to associate Dnmt expression with disease risk by evaluating recurrence of pre-cancer, progression to invasive cancer or survival. Because these patients were not actively

followed by our institution, unfortunately we were unable to get this very important data related to disease risk. For other cancers (example, hepatocellular carcinoma), increased expression of DNMT1 and a parallel increase in the number of methylated genes and poor survival has been reported (16). Therefore, reporting a folate fortification associated change in Dnmt 1 in cervical tissues is an important preliminary finding.

Another limitation of our study is that at an individual subject level, we have no 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 there is no question that serum folate concentrations have significantly increased after folic acid fortification (17). Although the lack of these data can be viewed as a limitation of the study, we believe that the results generated by this study are important preliminary findings that raise concerns about possible adverse effects of folate fortification on cancer. Finally, because this is the first study to report a folate fortification associated change in Dnmt 1, validating these results in other study populations or with other techniques of assessing Dnmt 1 (eg; Q RT PCR or western blot) will increase the scientific credibility of these findings.

Acknowledgements

Authors wish to thank Ms. Ilene Brill (systems analyst) for her assistance in data analysis.

Supported by Grant R03 CA 102893 from the National Cancer Institute

References

1. Piyathilake CJ, Azrad M, Jhala D, Macaluso M, Kabagambe EK, Brill I, Niveleau A, Jhala N, Grizzle WE. Mandatory fortification with folic acid in the United States is not associated with changes in the degree or the pattern of global DNA methylation in cells involved in cervical carcinogenesis. Cancer Biomark 2006;2(6):259–66. [PubMed: 17264397]
2. Reed MC, Nijhout HF, Neuhouser ML, Gregory JF 3rd, James SJ, Boynton A, Ulrich CM. A mathematical model gives insights into nutritional and genetic aspects of folate-mediated one-carbon metabolism. J Nutr 2006;136(10):2653–66. [PubMed: 16988141]
3. Basten GP, Duthie SJ, Pirie L, Vaughan N, Hill MH, Powers HJ. Sensitivity of markers of DNA stability and DNA repair activity to folate supplementation in healthy volunteers. Br J Cancer 2006 Jun 19;94 (12):1942–7. [PubMed: 16736000]
4. Lee WJ, Zhu BT. Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols. Carcinogenesis 2006;27(2):269–77. [PubMed: 16081510]
5. Grizzle, WE.; Myers, RB.; Manne, U.; Srivastava, S. Immunohistochemical evaluation of biomarkers in prostate and colorectal neoplasia. In: Hanausek, M.; Walaszek, Z., editors. John Walker's Methods in Molecular Medicine - Tumor Marker Protocols. New Jersey; Humana Press: 1998. p. 143-160.
6. Piyathilake, CJ.; Niveleau, A.; Grizzle, WE. Immunohistochemistry of in situ Hybridization of Human Carcinomas, Vol. 1: Molecular Genetics; Lung and Breast Carcinomas. Graphic World Publishing Service; Role of global DNA methylation in lung carcinoma.
7. Geiman TM, Robertson KD. Chromatin remodeling, histone modifications, and DNA methylation - how does it all fit together? J Cell Biochem 2002;87:117–125. [PubMed: 12244565]
8. Klimasauskas S, Roberts RJ. M. HhaI binds tightly to substrates containing mismatches at the target site. Nucl Acids Res 1995;23:1388–1395. [PubMed: 7753630]
9. Yang AS, Shen J-C, Zingg J-M, Mi S, Jones PA. Hha and HpaII DNA methyltransferases bind DNA mismatches, methylate uracil and block DNA repair. Nucleic Acids Res 1995;23:1380–1386. [PubMed: 7753629]
10. James SJ, Pogribny IP, Pogribna M, Miller BJ, Jernigan S, Melnyk S. Mechanisms of DNA damage, DNA hypomethylation, and tumor progression in the folate/methyl-deficient rat model of hepatocarcinogenesis. J Nutr 2003 Nov;133(11 Suppl 1):3740S–3747S. [PubMed: 14608108]

11. Laird PW, Jaenisch R. DNA methylation and cancer. Hum Mol Genet 1994;3:1487–1495. [PubMed: 7849743]
12. Kim YI. Will mandatory folic acid fortification prevent or promote cancer? Am J Clin Nutr 2004;80:1123–8. [PubMed: 15531657]
13. Kim YI. Does a high folate intake increase the risk of breast cancer? Nutr Rev 2006;64(10 Pt 1):468–75. [PubMed: 17063929]
14. van Engeland M, Weijenberg MP, Roemen GM, et al. Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands Cohort Study on Diet and Cancer. Cancer Res 2003;63:3133–7. [PubMed: 12810640]
15. Kawakami K, Ruszkiewicz A, Bennett G, Moore J, Watanabe G, Iacopetta B. The folate pool in colorectal cancers is associated with DNA hypermethylation and with a polymorphism in methylenetetrahydrofolate reductase. Clin Cancer Res 2003;9:5860–5. [PubMed: 14676107]
16. Oh BK, Kim H, Park HJ, Shim YH, Choi J, Park C, Park YN. DNA methyltransferase expression and DNA methylation in human hepatocellular carcinoma and their clinicopathological correlation. Int J Mol Med 2007;20(1):65–73. [PubMed: 17549390]
17. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 1999;340(19):1449–54. [PubMed: 10320382]

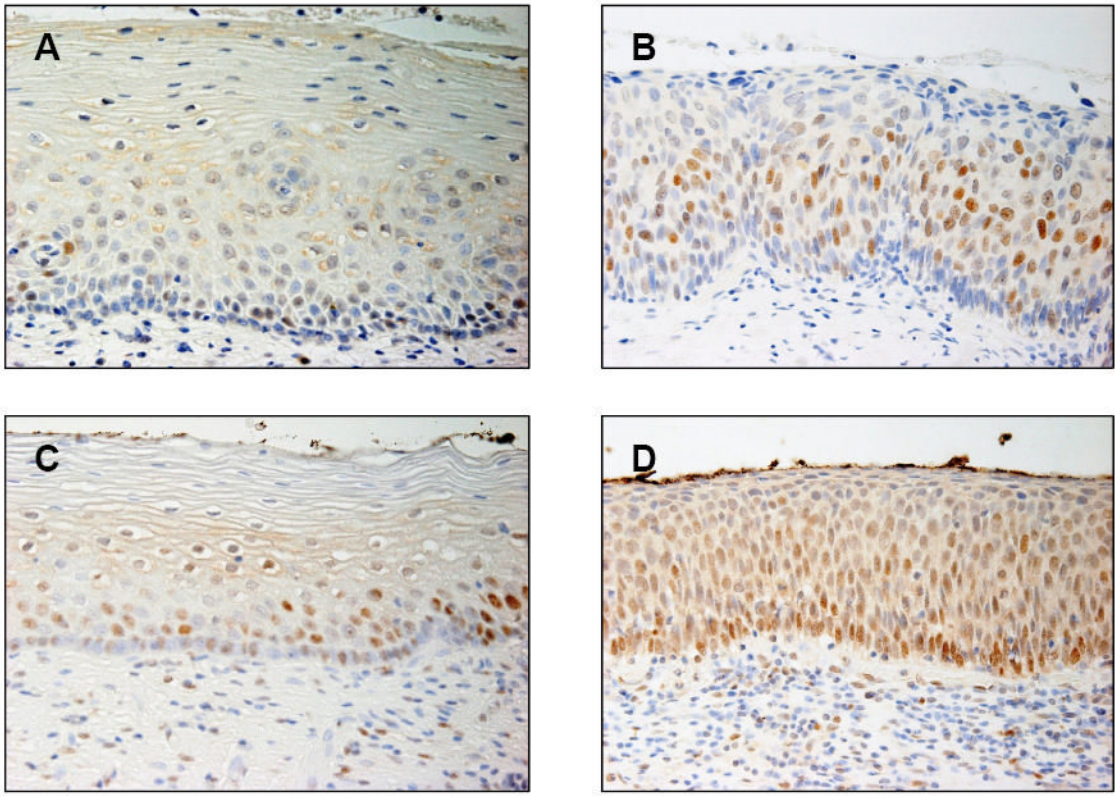

Figure 1.
Expression of Dnmt 1 in pre-fortification normal cervical epithelium (A); pre-fortification CIN 3 lesion (B); post-fortification normal cervical epithelium (C) and post-fortification CIN 3 lesion (D)

Table 1

The expression of Dnmt 1 in the pre-and post-fortification periods

	Pre-fortification (1990-1992)			Post-fortification (2000-2002)			
	AA	CA	AA + CA	AA	CA	AA + CA	
Number of subjects	14	32	46	10	28	38	
Age, years (Mean ±SD)	34.36±14.29	34.31±13.39	34.33±13.51	31.30±7.02	31.89±9.44	31.74±8.78	
% cells positive for DNMT*							p†
≤ CIN 1	12.04±7.37	12.48±2.98	12.26±4.03	52.22±8.59	49.28±5.34	50.75±5.04	<.0001
≥ CIN 2	30.59±7.99	29.58±4.97	30.09±4.70	83.35±4.73	75.52±4.23	79.44±3.14	<.0001
Intensity score for DNMT*							
≤ CIN 1	0.15±0.09	0.15±0.04	0.15±0.05	0.64±0.11	0.63±0.07	0.63±0.06	<.0001
≥ CIN 2	0.42±0.11	0.40±0.06	0.41±0.07	1.15±0.10	1.08±0.08	1.11±0.06	<.0001

AA = African Americans; CA = Caucasian Americans; CIN = Cervical Intraepithelial Neoplasia; CIS = Carcinoma *in situ*

*Values are means ± SE, adjusted for age and race

†P comparing AA + CA for pre- and post fortification