Published in final edited form as:
Nutrition. 2011 May 1; 27(5): 513–519. doi:10.1016/j.nut.2010.08.018.

A higher degree of LINE-1 methylation in peripheral blood mononuclear cells, a one-carbon nutrient related epigenetic alteration, is associated with a lower risk of developing cervical intraepithelial neoplasia

Chandrika J. Piyathilake[1], **Maurizio Macaluso**[2], **Ronald D. Alvarez**[3], **Min Chen**[1], **Suguna Badiga**[1], **Nuzhat R Siddiqui**[1], **Jeffrey C Edberg**[4], **Edward E Partridge**[5], and **Gary L Johanning**[6]

[1]The Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, Alabama

[2]The Department of Epidemiology, University of Alabama at Birmingham, Birmingham, Alabama

[3]The Department of Obstetrics & Gynecology, University of Alabama at Birmingham, Birmingham, Alabama

[4]The Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

[5] Comprehensive Cancer Center of the University of Alabama at Birmingham, Birmingham, Alabama

[6] Department of Veterinary Sciences of the The University of Texas MD Anderson Cancer Center.

Abstract

Objective—The objective of the study was to evaluate LINE-1 methylation as an intermediate biomarker for the effect of folate and vitamin B12 on the occurrence of higher grades of cervical intraepithelial neoplasia (CIN 2+).

Methods—Study included 376 women who tested positive for HR-HPVs and were diagnosed with CIN 2+ (cases) or ≤ CIN 1 (non-cases). CIN 2+ (yes/no) was the dependent variable in logistic regression models that specified the degree of LINE-1 methylation of peripheral blood mononuclear cells (PBMCs) and of exfoliated cervical cells (CCs) as the independent predictors of primary interest. In analyses restricted to non-cases, PBMC LINE-1 methylation (≥70% vs. <70%) and CC LINE-1 methylation (≥54% vs. <54%) were the dependent variables in logistic regression models that specified the circulating concentrations of folate and vitamin B12 as the primary independent predictors.

Results—Women in the highest tertile of PBMC LINE-1 methylation had 56% lower odds of being diagnosed with CIN 2+ (OR = 0.44; 95% CI, 0.24-0.83; *P* = 0.011) while there was no significant association between degree of CC LINE-1 methylation and CIN 2+ (OR = 0.86; 95%

Correspondence to: Chandrika J Piyathilake, Department of Nutrition Sciences, University of Alabama at Birmingham (UAB), 1675 University Blvd, Webb 326, Birmingham, AL 35294. Phone: 205-975-5398 Fax: 205-966-2859 piyathic@uab.edu.

CI, 0.51-1.46; $P = 0.578$). Among non-cases, women with supra-physiologic concentrations of folate (>19.8 ng/mL) and sufficient concentrations of plasma vitamin B12 (≥ 200.6 ng/mL) were significantly more likely to have highly methylated PBMCs compared to women with lower folate and lower vitamin B12 (OR = 3.92; 95% CI, 1.06-14.52; $P = 0.041$). None of the variables including folate and vitamin B12 were significantly associated with CC LINE-1 methylation.

Conclusions—These results suggest that a higher degree of LINE-1 methylation in peripheral blood mononuclear cells, a one-carbon nutrient related epigenetic alteration, is associated with a lower risk of developing cervical intraepithelial neoplasia.

Keywords

methylation; cervical; neoplasia

Introduction

Biomarkers that are influenced by dietary factors may help explain the effects of those factors on cancer risk and assist in monitoring the effectiveness of dietary interventions. Aberrant DNA methylation, one of the most common molecular alterations in human cancer is shown to be associated with carcinogenic processes (1), but little is known about the relationship between this epigenetic alteration and diet or other lifestyle factors.

The studies we conducted at the beginning of the US folic acid fortification program (1996-1998) demonstrated that higher circulating concentrations of folate can reduce the impact of high-risk (HR) human papillomaviruses (HPVs) on the risk of developing cervical intraepithelial neoplasia (CIN), the precursor of invasive cervical cancer (2,3). Our more recent studies, conducted a few years after the US folic acid fortification had started (2004-2006) demonstrated that supra-physiological concentrations of plasma folate are associated with significantly lower risk of CIN, especially when vitamin B12 is sufficient, demonstrating the importance of vitamin B12 in the high folate environment created by the US folic acid fortification program (4). DNA methylation is a possible biomarker for the effect of folate and vitamin B12 on CIN risk. Evaluation of the influence of folate on DNA methylation is also important because concerns have been raised about population-wide exposure to higher levels of folic acid (synthetic form of folate) in the post-folic acid fortification era i.e. cognitive impairment and anemia, immune function, reducing the efficacy of anti-epileptic drugs or anti-folate chemotherapy or promoting the progression of initiated cancer cells. (5,6,7,8),

We chose to evaluate the degree of methylation in the LINE-1 promoter using pyrosequencing because this technique is preferable to COBRA, MsSNuPE or MethyLight for the purpose of measuring changes in LINE methylation (9). It has been hypothesized that DNA methylation evolved as a defense mechanism against DNA pathogens as a way to silence foreign DNA sequences (10,11). Thus, a lower degree of LINE-1 methylation may play a role in modifying the risk of HPV-associated cancer. In addition to changes in LINE-1 methylation in target cells (in our case, cervical epithelial cells), changes in LINE-1 methylation in peripheral blood mononuclear cells (PBMCs) may also alter cancer risk through an effect on immune response-related genes.

The main objectives of the current study were 1) to evaluate the association of LINE-1 methylation in cervical cells (CCs) and in PBMCs with CIN 2+ and 2) to assess nutritional determinants of CC and PBMC LINE-1 methylation.

Materials and Methods

Patient population

The present analysis is based on women (n=376) enrolled in an ongoing prospective follow-up study funded by the National Cancer Institute (R01 CA105448, Prognostic Significance of DNA & Histone Methylation). The study has been described in a previous publication (4). All women were diagnosed with abnormal cervical cells in clinics of the Health Departments in Jefferson County and surrounding counties in Alabama and were referred to the University of Alabama at Birmingham (UAB) for further examination by colposcopy and biopsy. Women were 19-50 years old, had no history of cervical cancer or other cancer of the lower genital tract, no history of hysterectomy or destructive therapy of the cervix; were not pregnant, and were not using antifolate medications such as methotrexate, sulfasalazine, or phenytoin. Of these 376 women, 103 women were diagnosed with CIN 2+ (cases, including CIN 2 [n=62], CIN 3 [n= 38] or carcinoma *in situ* [CIS, n= 3]) and 273 women were diagnosed with ≤ CIN 1 (non-cases, including normal cervical epithelium [n= 13], HPV cytopathic effect [HCE, n= 31], reactive nuclear enlargement [RNE, n=48] or CIN 1 [n=181]). Both cases and controls tested positive for HR-HPV (any one of 13 types of HR-HPV, HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). All women included in this analysis participated in an interview that assessed socio-demographic variables and lifestyle risk factors (questionnaire developed at the clinic) and physical activity (CDC questionnaire). Height, weight and waist circumference (WC) measurements were obtained using standard protocols. The BMI was calculated using the height and weight measurements (weight kg/[height m]^2). Pelvic examinations and collection of cervical cells and biopsies were carried out following the protocols of the colposcopy clinic. Fasting blood samples were collected from all women. The study protocol and procedures were approved by the UAB Institutional Review Board.

Laboratory Methods

Exfoliated CCs were collected from the transformation zone with a cervical brush and immediately rinsed in 10 ml of phosphate buffered saline (PBS). The fasting blood samples were collected in EDTA containing blood collection tubes and kept on ice until they were transported to the laboratory within two hours from collection. In the laboratory, CC suspensions were centrifuged and the resulting pellets were re-suspended in fresh PBS. CC aliquots used for HPV genotyping were stored in PreservCyt Solution at -20°C while CC aliquots used for LINE-1 methylation were stored at -80°C. Blood samples were processed to isolate plasma, buffy coat and red blood cells. All components were stored at -80°C including the buffy coat used to extract DNA for methylation analysis. DNA was extracted from cervical cells and buffy coats using a standard phenol-chloroform extraction method. As described below, methylation analysis of LINE-1 promoter (GeneBank accession no.x58075) in CCs and in PBMCs was investigated using a pyrosequencing-based methylation analysis.

Bisulfite-pyrosequencing LINE-1 analysis

Bisulfite treatment of 1 μg of DNA extracted from each sample was completed using the EZ DNA methylation kit (Zymo Research, CA) and the converted DNA was eluted with 30 μl TE buffer. The bisulfite-modified DNA was stored at -80 C until used. A reaction volume of 25 μl was amplified by PCR. Each reaction mixture contained 5 μl of bisulfite-modified DNA, 250 μM dNTP, 0.25 μM of the forward primer (5'-TTTTTTGAGTTAGGTGTGGG-3'), 0.25 μM of the reverse-biotinylated primer (5'-biotin-TCTCACTAAAAAATACCAAACAA-3') (12), 0.25 mM $MgCl_2$ and 0.025 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR was carried out in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). PCR cycling

conditions were 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 45s, 50°C for 40s, and 72°C for 45s, and a final extension of 1 cycle of 72°C for 5 min. The biotinylated PCR product was purified and made single-stranded to act as a template, using the Pyrosequencing Vacuum PrepTool (Biotage, Inc. Charlottesville, VA). In brief, 10 μl of each biotinylated PCR product was immobilized on streptavidin-coated Sepharose HP beads (Amersham Biosciences, Piscataway, NJ) and the non-biotinylated strand was removed using 0.2 M NaOH. The biotinylated single stranded product was annealed to the pyrosequencing primer (5'- GGGTGGGAGTGAT-3') (0.4μM final concentration), and then was subjected to sequencing using an automatically generated nucleotide dispensation order for sequences to be analyzed corresponding to each reaction. The pyrograms were analyzed using allele quantification (AQ) mode to determine the proportion of C/T, and hence methylated and unmethylated cytosines at the targeted position(s). The degree of methylation was evaluated at three CpG methylation sites. Reproducibility of the assay was satisfactory with a CV of 2-2.2%.

Determination of circulating concentrations of micronutrients

Circulating concentrations of micronutrients were determined using protocols previously established and validated in the Nutrition Sciences Laboratory at the University of Alabama at Birmingham (13). Briefly, plasma folate was measured using the *L. Casei* microbiological assay, plasma vitamin B-12 using a competitive radio-binding assay (SimulTRAC-SNB, MP Biomedicals), vitamins A, E, and C by high performance liquid chromatography [HPLC], and total carotene by spectrophotometry. All samples were stored at -80°C until micronutrient assays were completed within 2-3 months of collection.

Statistical analysis

We used descriptive statistics to characterize the 103 cases and 273 non-cases. The Pearson chi-square statistic was used to assess differences between observed and expected frequencies. Proportions were compared using a two-sided chi-square test. The differences in median concentrations of micronutrients between cases and controls were compared using the two-sided Kruskal-Wallis test. The degree of LINE-1 methylation is reported as the average percent methylation of the three CpG sites evaluated. We determined the correlation between PBMC LINE-1 methylation and CC LINE-1 methylation for the entire study population and stratified by case status using Spearman's rank correlation. Unconditional logistic regression models specified a binary indicator of CIN 2+ diagnosis (yes/no) as the dependent variable. We examined the degree of PBMC and CC LINE-1 methylation as a potential biomarker for CIN status after adjusting for age (median), race (African American vs. Caucasian American), education (less than high school education vs. high school education or higher), smoking status (current smoker vs. non-current smoker), parity (> 1 live birth vs. 0 births), hormone contraceptive use (ever vs. never), degree of physical activity (≥ 150 min/week moderate activity vs. < 150 min/week moderate activity), WC (> 88 cm vs. ≤ 88 cm)/BMI (>25 vs. ≤ 25) and all circulating concentrations of micronutrients (tertiles) as categorical variables. We categorized the percent PBMC LINE-1 methylation and CC LINE-1 methylation based on the highest tertile (≥ 70% & ≥ 54% respectively). Folate and vitamin B12 levels were not included in the model presented, as they were considered *a priori* determinants of PBMC and CC LINE-1 methylation, and would have been inappropriate to include such distal determinants of the outcome in the same regression model. Analyses including folate and vitamin B12 levels were presented in a previous publication (4) and showed a significant combined protective effect of supra-physiologic levels of folate (>19.8 ng/mL) and sufficient vitamin B12 (≥ 200.6 pg/mL) on CIN 2+ risk. Both models were run replacing WC with BMI. Additional analyses were restricted to women with PBMC LINE-1 methylation <70% to ascertain whether the combined

protective effect of folate and vitamin B12 was present in the absence of higher LINE-1 methylation.

Determinants of PBMC LINE-1 methylation and CC LINE-1 methylation were analyzed among non-cases. Exclusion of the cases was necessary to avoid the possibility of reverse causation (i.e., CIN 2+ status influencing PBMC or CC LINE-1 methylation). Unconditional logistic regression models were used to examine the association between a binary indicator of percent PBMC LINE-1 methylation (≥70% vs. <70%) or CC LINE-1 methylation (≥ 54% vs. <54%), and circulating concentrations of folate and vitamin B12, independent predictors of primary interest, after adjusting for age, race, education level, smoking status, parity, degree of physical activity, WC/BMI and circulating concentrations of other micronutrients (vitamins A, C, gamma tocopherol, and total carotene). We analyzed this association by comparing three categories: 1) supra-physiologic concentrations of plasma folate (>19.8 ng/mL) and sufficient concentrations of plasma vitamin B12 (≥ 200.6 ng/mL), 2) supra-physiologic concentrations of plasma folate (>19.8 ng/mL) and plasma vitamin B12 <200.6 ng/mL, and 3) plasma folate ≤19.8 ng/mL and plasma vitamin B12 <200.6 ng/mL, used as the reference category. Only two subjects with available data were in the combination of folate >19.8 ng/mL and plasma vitamin B12 <200.6 ng/mL, and were excluded from the analysis. We categorized other circulating micronutrients into tertiles and included the tertiles as a continuous variable in the model to account for a linear trend. Both models were run replacing WC with BMI. We evaluated the strength and precision of each association by estimating the odds ratio (OR) and its 95% confidence interval (CI), and its statistical significance using Wald's chisquare test of the null hypothesis that the OR=1. All statistical analyses were conducted using SAS® Version 9.1.3 (SAS Institute, Cary, NC).

Results

All women were of premenopausal age, and all except three were younger than 45 years of age. Compared to the 273 non-cases, the 103 cases were significantly less likely to be African-American ($P = 0.04$), and more likely to be current smokers ($P = 0.002$). Parity was significantly higher in cases than in non-cases ($P = 0.017$); and non-cases were more frequently nulliparous than cases. Compared to the non-cases, fewer cases had sufficient plasma vitamin B12 concentrations (≥ 200.6 pg/mL) ($P = 0.032$) and supra-physiologic plasma folate concentrations (>19.8 ng/mL), although the latter difference was not statistically significant.

The degree of LINE-1 methylation was highest at site 1 and lowest at site 2 in both PBMCs and CCs, and was higher in PBMCs than in CCs at all three sites (Table 1). The average PBMC LINE-1 methylation tended to be lower in cases than in non-cases ($P = 0.09$), while the average CC methylation was similar in the two groups ($P = 0.58$). The cases had a narrower range of PBMC methylation (Table 1).

No significant correlation was observed between LINE-1 methylation in PBMCs and CCs of the entire study population (r = 0.06; $P = 0.25$). However, when the population was stratified by case status, a weak correlation between PBMC LINE-1 methylation and CC LINE-1 methylation was observed in non-cases which approached significance (r = 0.12; $P = 0.06$) and no significant correlation was observed in cases (r = 0.06; $P = 0.31$).

Women in the highest tertile of PBMC LINE-1 methylation (≥70%) were 56% less likely to be diagnosed with CIN 2+ than women in the lower tertiles (P=0.011) (Table 2). There was no significant association between degree of CC LINE-1 methylation and prevalence of CIN 2+. Because in these analyses DNA methylation is evaluated as an intermediate marker of the effect of folate and vitamin B12, these variables were not included in the regression

model. To assess whether the effect of folate and vitamin B12 was entirely mediated by higher PBMC LINE-1 methylation, separate analyses were restricted to women with PBMC LINE-1 methylation <70%. The results of these analyses (not shown in detail) revealed a residual effect of folate and vitamin B12 that was no longer statistically significant.

We evaluated the combined effects of folate and vitamin B12 on LINE-1 methylation among non-cases after adjusting for other demographic and lifestyle factors and circulating concentrations of other cancer associated micronutrients (Table 3). Women who engaged in moderate physical activity for ≥150 minutes/week and women with higher circulating concentrations of gamma-tocopherol were less likely to have highly methylated PBMCs. Women with higher circulating concentrations of vitamin C were more likely to have highly methylated PBMCs, but this association did not achieve statistical significance ($P = 0.061$). Women with supra-physiologic concentrations of folate and sufficient vitamin B12 were significantly more likely to have highly methylated PBMCs compared to women with lower folate and lower vitamin B12 (OR = 3.92; 95% CI, 1.06-14.52; $P = 0.041$). None of these variables, including folate and vitamin B12, were significantly associated with CC LINE-1 methylation. Replacing WC with BMI did not alter the results.

Discussion

DNA methylation is a major epigenetic mechanism of controlling gene expression in mammalian cells (14,15). Even though DNA methylation changes in human cancers are characterized by generalized genomic hypomethylation and hypermethylation of focal CpG islands, and both events are able to alter gene expression, emphasis has been largely focused on functional significance of hypermethylation in CpG islands on promoters (16). Because of this intense focus on hypermethylation events, the role of genome-wide DNA hypomethylation in human cancer has been under-studied. Recent studies have focused on repetitive DNA elements such as *LINE-1* and *SAT2* that are located throughout the human genome. These elements remain heavily methylated in normal cells, and their methylation levels, as measured by PCR-based methods has been shown to correlate well with overall genomic 5-methylcytosine content (17) indicating that analysis of Alu and LINE-1 methylation serves as a surrogate measure of genomic methylation levels. LINE repeat regions located on specific chromosomes (e.g., 22q11-q12) are shown to be a consistent indicator of global methylation status (18,19). Yang et al. demonstrated that changes in the methylation levels of abundant LINE-1 repetitive elements (GeneBank accession no. x58075) could also be used as a surrogate marker of genome-wide methylation changes (20). Studies have shown that methylation levels measured in these abundant LINE-1 regions, which are easy to characterize by pyrosequencing technology, do not vary significantly with time within an individual, and therefore changes in their methylation levels could potentially be attributed to dietary or lifestyle factors or interventions with such factors (9). In addition to LINE-1 genome-wide methylation assays, gene-specific assays are also used to assess the influence of folate status on gene methylation. In one such study, there was no effect of high serum folate levels on methylation patterns of the ec-SOD gene promoter in healthy subjects (21).

Hypomethylation-mediated reactivation of active forms of LINEs (LINE-1) in relation to human cancer risk has been under investigation for several years. LINE-1 retrotransposons are shown to be hypomethylated in many cancers (22,23,24) suggesting potential activation of LINE-1 in these cancers. Based on these observations, it is biologically plausible that reactivation of LINE-1 elements through hypomethylation could potentially induce genomic instability, thus transforming these cells into a precancerous or cancerous state. In support of this, studies have shown that LINE-1 retrotransposition is able to induce DNA damage and apoptosis in a breast cancer cell lines (25). In addition, hypomethylation of LINE-1 elements

are shown to be associated with increased genomic instability in non-small cell lung cancer (26) and colorectal carcinomas (12).

In addition to the genome-wide hypomethylation in cancer tissues, results support the notion that degree of LINE-1 methylation in "normal" cells such as peripheral blood derived DNA is a biomarker for susceptibility for head and neck squamous cell carcinoma (27). This study assessed whole blood global DNA methylation levels in one long interspersed nuclear element repeat region, *LRE1*, located on 22q11-q12 using a modified version of the combined bisulfite restriction analysis. An inverse association between leukocyte global DNA methylation (assessed by using a combination of high performance capillary electrophoresis (HPCE) and Hpa II digestion of DNA) and bladder cancer has been reported previously (28). A recent study suggested that leukocyte DNA hypomethylation determined by liquid chromatography-electrospray ionization tandem mass spectrometry is independently associated with development of breast cancer (29). Genomic methylation of leukocyte DNA determined by liquid chromatography-mass spectrometry is also shown as a potential etiologic factor for early stage of colorectal adenomas (30). Reverse causation is theoretically possible in cross-sectional studies of cancer, which is the presence of cancer may lower leukocyte DNA methylation, but it is unlikely that this occurs with precancerous lesions such as adenomas or, as is the case of this study, CIN2+. No previous studies have been able to rule out this possibility because of lack of methylation data in peripheral cells and in cancer or pre-cancer cells of the same individuals. We addressed this gap in knowledge by measuring LINE-1 methylation in paired samples of PBMCS and CCs in our study population.

Our results showed that only the degree of PBMC LINE-1 methylation is independently associated with CIN 2+. Lack of significant correlation between PBMC and CC LINE-1 methylation indicates that PBMC methylation is not a surrogate marker for CC methylation. Higher LINE-1 methylation of PBMCs and its association with lower likelihood of CIN 2+ diagnosis may be related to the ability of those individuals to mount a protective immune response to HR-HPVs. The study of Hsiung et al supports this idea by demonstrating that PBMC methylation has a positive influence on immune response against HPV related head and neck cancer (31). Therefore, our results suggest that susceptibility to HR-HPV related cervical carcinogenesis may also be an epigenetically modified process operating via a central mechanism rather than via a mechanism operating at target tissues. Immune response has been shown to play a crucial role in eliminating HPV infections (32). Cell-mediated immune responses, especially Th1 responses leading to increased IL-2 and IFN- production, are likely to be the major component that contributes to the development of cell-mediated immunity against HPV infections and HPV-associated neoplasms (33). The wider range of immune responses demonstrated in these studies, however, suggests the possibility that host factors such as nutritional status may modify the immune responsiveness at an individual level. In this regard, β-hydroxy-β-methylbutyrate, which is an intermediate of leucine metabolism, impaired lymphocyte proliferation and progression through the cell cycle and modified the Th1/Th2 cytokine production towards a Th2 profile when HMB was present at 10 mM (34). Several lines of evidence from animal studies suggested that folate deficiency affects cell-mediated immune functions (35,36) and supplementation with folic acid is able to improve the immune response (37).

We observe that the combination of supra-physiologic concentrations of folate and sufficient vitamin B12 has a positive influence on PBMC LINE-1 methylation and higher PBMC methylation is associated with lower risk of CIN 2+. Therefore, our current observations strengthen our previous finding that supra-physiologic plasma folate concentrations seen in the post US folic acid fortification era do not increase the risk of CIN in premenopausal women of childbearing age, especially in the presence of sufficient vitamin B12 status,

because the same micronutrient combination is able to exert a positive influence on PBMC methylation which in turn reduces the risk of CIN 2+. However, these results do not exclude the possibility of adverse effects of exposure to higher folate on other health conditions in this population.

In addition to folate and vitamin B12, we observe that other lifestyle factors and micronutrients may have an influence on PBMC methylation. Higher physical activity seems to be associated with a lower degree of PBMC DNA methylation, but interpretation of this finding is unclear. More extensive data on physical activity rather than self-reported data may be needed to explain these contradictory findings. Higher vitamin C is positively associated with PBMC LINE-1 methylation, while gamma-tocopherol is inversely associated. In our previous studies, these two micronutrients were not independently associated with CIN risk (4). Therefore, it is unlikely that these micronutrients have significant influence on CIN risk via PBMC methylation.

In summary, even though several biological mechanisms by which folate could modify the risk associated with HR-HPV are highly plausible and include folate's effects on cancer biomarkers such as methylation of DNA and histones and DNA damage in target cells, or folate's effects on immune responses, all of these are largely unproven mechanisms. We propose PBMC LINE-1 methylation as a possible intermediate biomarker for the effect of folate and vitamin B12 on the occurrence of CIN 2+ in our study population. Our previous studies documented a reduced prevalence of CIN 2+ among women with supra-physiologic folate and sufficient vitamin B12 levels (4). In this report, we show that increased PBMC LINE-1 methylation is strongly associated with supra-physiologic concentrations of folate and sufficient vitamin B12 concentrations among non-cases, suggesting that higher folate and vitamin B12 affect PBMC LINE-1 methylation before the development of CIN. In addition, in the comparison of cases and non-cases, PBMC LINE-1 methylation is a strong predictor of decreased CIN risk. These findings are consistent with the hypothesis that PBMC LINE-1 methylation mediates the effects of folate and vitamin B12 in the pathway leading to CIN in women exposed to HR-HPVs. Documenting causal pathways in epidemiologic analyses is difficult, and our analysis of this cross-sectional study cannot conclusively address the questions of whether PBMC LINE-1 methylation is a real intermediary of the protective effect of folate and vitamin B12, and whether other pathways exist. The presence of a residual, non-significant protective effect of folate and vitamin B12 among women with <70% PBMC LINE-1 methylation is consistent with other mechanisms for the effect of the two micronutrients, but cannot exclude that PBMC LINE-1 methylation is the only pathway. The results from our ongoing follow-up study may provide both a more appropriate temporal relation, and more adequate numbers to address these issues.

Acknowledgments

Supported by R01 CA105448 funded by the National Cancer Institute

References

1. Piyathilake CJ, Frost AR, Bell WC, et al. Altered global methylation of DNA: an epigenetic difference in susceptibility for lung cancer is associated with its progression. Hum Pathol. 2001; 32(8):856–862. [PubMed: 11521231]
2. Piyathilake CJ, Henao OL, Macaluso M, et al. Folate is associated with the natural history of high-risk human papillomaviruses. Cancer Res. 2004; 64(23):8788–8793. [PubMed: 15574793]
3. Piyathilake CJ, Macaluso M, Brill I, et al. Lower red blood cell folate enhances the HPV-16-associated risk of cervical intraepithelial neoplasia. Nutrition. 2007; 23(3):203–210. [PubMed: 17276035]

4. Piyathilake CJ, Macaluso M, Alvarez RD, et al. Lower risk of cervical intraepithelial neoplasia in women with high plasma folate and sufficient vitamin B12 in the post-folic acid fortification era. Cancer Prev Res (Phila Pa). 2009; 2(7):658–664.
5. Smith AD, Kim YI, Refsum H. Is folic acid good for everyone? Am J Clin Nutr. 2008; 87(3):517–533. [PubMed: 18326588]
6. Kim YI. Folic acid fortification and supplementation--good for some but not so good for others. Nutr Rev. 2007; 65(11):504–511. [PubMed: 18038943]
7. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B12 status in relation to anemia, macrocytosis, and cognitive impairment among older Americans in the age of folic acid fortification. Am J Clin Nutr. 2007; 85:193–200. [PubMed: 17209196]
8. Troen AM, Mitchell B, Sorensen B, Wener MH, Johnston A, Wood B, Selhub, McTiernan A, Yasui Y, Oral E, Potter JD, Ulrich CM. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr. 2006; 136:189–94. [PubMed: 16365081]
9. Aparicio A, North B, Barske L, et al. LINE-1 methylation in plasma DNA as a biomarker of activity of DNA methylation inhibitors in patients with solid tumors. Epigenetics. 2009; 4(3):176–184. [PubMed: 19421002]
10. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet. 1997; 13(8):335–340. [PubMed: 9260521]
11. Bird AP. Functions for DNA methylation in vertebrates. Cold Spring Harb Symp Quant Biol. 1993; 58:281–285. [PubMed: 7956040]
12. Estécio MR, Gharibyan V, Shen L, et al. LINE-1 Hypomethylation in Cancer Is Highly Variable and Inversely Correlated with Microsatellite Instability. PLoS ONE. 2007; 2(5):e399. [PubMed: 17476321]
13. Piyathilake CJ, Macaluso M, Hine RJ, et al. Local and systemic effects of cigarette smoking on folate and vitamin B12. Am J Clin Nutr. 1994; 60(4):559–566. [PubMed: 8092091]
14. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. Cell. 2007; 128(4):669–681. [PubMed: 17320505]
15. Egger G, Liang G, Aparicio A, et al. Epigenetics in human disease and prospects for epigenetic therapy. Nature. 2004; 429(6990):457–463. [PubMed: 15164071]
16. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003; 349(21):2042–2054. [PubMed: 14627790]
17. Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res. 2005; 33(21):6823–6836. [PubMed: 16326863]
18. Ostertag EM, Kazazian HH Jr. Biology of mammalian L1 retrotransposons. Ann Rev Genet. 2001; 35:501–538. [PubMed: 11700292]
19. Chalitchagorn K, Shuangshoti S, Hourpai N, et al. Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. Oncogene. 2004; 23(54):8841–8846. [PubMed: 15480421]
20. Yang AS, Estecio MR, Doshi K, et al. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res. 2004; 32(3):e38. [PubMed: 14973332]
21. Hirsch S, Ronco AM, Guerrero-Bosagna C, de la Maza MP, Leiva L, Barrera G, Llanos M, Alliende MA, Silva F, Bunout D. Methylation status in healthy subjects with normal and high serum folate concentration. Nutrition. Nov-Dec; 2008 24(11-12):1103–9. [PubMed: 18653314]
22. Suter CM, Martin DI, Ward RL. Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. Int J Colorectal Dis. 2004; 19(2):95–101. [PubMed: 14534800]
23. Cho NY, Kim BH, Choi M, et al. Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. J Pathol. 2007; 211(3):269–277. [PubMed: 17139617]
24. Pattamadilok J, Huapai N, Rattanatanyong P, et al. LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. Int J Gynecol Cancer. 2008; 18(4):711–717. [PubMed: 17944913]

25. Belgnaoui SM, Gosden RG, Semmes OJ, Haoudi A. Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. Cancer Cell Int. 2006; 6:13. [PubMed: 16670018]
26. Daskalos A, Nikolaidis G, Xinarianos G, et al. Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. Int J Cancer. 2009; 124(1):81–87. [PubMed: 18823011]
27. Ting Hsiung D, Marsit CJ, Houseman EA, et al. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev. 2007; 16(1):108–114. [PubMed: 17220338]
28. Wentzensen N, Sherman ME, Schiffman M, et al. Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. Gynecol Oncol. 2009; 112(2):293–299. [PubMed: 19054549]
29. Choi JY, James SR, Link PA, et al. Association between global DNA hypomethylation in leukocytes and risk of breast cancer. Carcinogenesis. Jul 7.2009 [Epub ahead of print].
30. Lim U, Flood A, Choi SW, et al. Genomic methylation of leukocyte DNA in relation to colorectal adenoma among asymptomatic women. Gastroenterology. 2008; 134(1):47–55. [PubMed: 18166347]
31. Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, Kelsey KT. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev. 2007; 16(1):108–114. [PubMed: 17220338]
32. Frazer IH, Tindle RW. Cell-mediated immunity to papillomaviruses. Papillomavirus Report. 1992; 3:53–58.
33. Wu TC, Kurman RJ. Analysis of cytokine profiles in patients with human papillomavirus-associated neoplasms. J Natl Cancer Inst. 1997; 89(3):185–186. [PubMed: 9016995]
34. Nunes EA, Lomax AR, Noakes PS, Miles EA, Fernandes LC, Calder PC. beta-Hydroxy-beta-methylbutyrate modifies human peripheral blood mononuclear cell proliferation and cytokine production in vitro. Nutrition. Jun 10.2010 [Epub ahead of print].
35. Williams EAJ, Gross RL, Newberne PM. Effect of folate deficiency on the cell-mediated immune response in rats. Nutr Rep Int. 1975; 12:137–148.
36. Gebhardt BM, Newberne PM. Nutrition and immunological responsiveness. T-cell function in the offspring of lipotrope and protein-deficient rats. Immunology. 1974; 26(3):489–495. [PubMed: 4137087]
37. Field CJ, Van Aerde A, Drager KL, Goruk S, Basu T. Dietary folate improves age related decrease in lymphocyte function. J Nutr Biochem. 2006; 17(1):37–44. [PubMed: 16098728]

Table 1

Degree of LINE- 1 methylation in PBMCs and in CCs by case status

PBMCs	Case status	Mean ± SD (%)	Median (%)	Range (%)
Site 1	Case	81 ± 4	82	24
	Non-case	82 ± 4	83	24
Site 2	Case	45 ± 9	41	40
	Non-case	46 ± 9	43	43
Site 3	Case	62 ± 9	59	46
	Non-case	64 ±10	62	65
Average*	Case	63 ± 7	60	37
	Non-case	64 ± 7	62	38
CCs				
Site 1	Case	73 ± 6	73	37
	Non-case	72 ± 8	72	68
Site 2	Case	33 ± 7	32	41
	Non-case	32 ± 8	32	70
Site 3	Case	52 ± 8	52	57
	Non-case	53 ±10	52	89
Average**	Case	53 ± 6	52	37
	Non-case	53 ± 8	52	57

* P=0.09 for average PBMC LINE-1 methylation between cases and non-cases

** P=0.58 for average CC LINE-1 methylation between cases and non-cases

Table 2

Relationship between Case-status and LINE-1 methylation

Risk factors	Model with PBMC* methylation		Model with CC** methylation	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Median age at enrollment (years)				
< 23	1.00 (referent)	0.391	1.00 (referent)	0.376
≥ 23	0.79 (0.46-1.36)		0.79 (0.46-1.34)	
Education				
High school education or greater	1.00 (referent)	0.432	1.00 (referent)	0.267
Less than high school education	0.77 (0.41-1.47)		0.70 (0.37-1.32)	
Race				
Caucasian American	1.00 (referent)	0.085	1.00 (referent)	0.092
African American	0.61 (0.34-1.07)		0.62 (0.35-1.08)	
Waist circumference				
≤ 88 cm	1.00 (referent)	0.471	1.00 (referent)	0.475
> 88 cm	0.83 (0.49-1.39)		0.83 (0.50-1.38)	
Moderate activity/week				
< 150 min	1.00 (referent)	0.049	1.00 (referent)	0.085
> 150 min	0.52 (0.27-0.996)		0.58 (0.31-1.08)	
Smoking status				
Non-current	1.00 (referent)	0.047	1.00 (referent)	0.077
Current	1.75 (1.01-3.05)		1.64 (0.95-2.83)	
Parity				
0 live birth	1.00 (referent)	0.032	1.00 (referent)	0.024
≥ 1 live births	1.96 (1.06-3.62)		2.02 (1.09-3.74)	
Life time sexual partners				
≤ 5	1.00 (referent)	0.476	1.00 (referent)	0.382
> 5	1.20 (0.72-2.00)		1.25 (0.76-2.05)	
Hormonal/oral contraceptive use				
Never user	1.00 (referent)	0.885	1.00 (referent)	0.683
Ever user	1.04 (0.63-1.72)		1.11 (0.68-1.82)	
LINE-1 methylation				
< highest tertile	1.00 (referent)	0.011	1.00 (referent)	0.578
≥ highest tertile	0.44 (0.24-0.83)		0.86 (0.51-1.46)	
Plasma micronutrients (tertiles)				
Vitamin C (μg/mL) <18.23, ≥18.23 - <12.4, ≥12.47	1.14 (0.84-1.55)	0.388	1.08 (0.80-1.46)	0.598
Gamma-tocopherol (mg %) <2.36, ≥2.36 - <1.72, ≥1.72	0.77 (0.56-1.05)	0.095	0.79 (0.58-1.07)	0.133
Vitamin A (μg %) <38.4,≥38.4 - <26.66, ≥26.66	0.99 (0.72-1.36)	0.933	0.96 (0.70-1.31)	0.800
Total carotene (μg %) <100.45, ≥100.45 - <72.61, ≥72.61	1.13 (0.83-1.55)	0.445	1.15 (0.84-1.57)	0.385

* Highest tertile of percent PBMC LINE-1 methylation is 70%

** Highest tertile of percent CC LINE-1 methylation is 54%

Table 3

Relationship of lifestyle factors, plasma folate/vitamin B12 combinations and other micronutrients with LINE-1 methylation in PBMCs and in CCs among non-cases

Risk factors	Model for PBMC* methylation		Model for CC** methylation	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Median age at enrollment (years)				
< 23	1.00 (referent)	0.839	1.00 (referent)	0.645
≥ 23	1.07 (0.56-2.07)		1.15 (0.64-2.07)	
Education				
High school education or greater	1.00 (referent)	0.709	1.00 (referent)	0.469
Less than high school education	1.16 (0.53-2.53)		0.76 (0.36-1.59)	
Race				
Caucasian American	1.00 (referent)	0.080	1.00 (referent)	0.440
African American	0.54 (0.27-1.08)		0.77 (0.40-1.49)	
Waist circumference				
≤ 88 cm	1.00 (referent)	0.958	1.00 (referent)	0.074
> 88 cm	0.98 (0.52-1.87)		1.73 (0.95-3.17)	
Moderate activity/week				
< 150 min	1.00 (referent)	0.001	1.00 (referent)	0.517
≥ 150 min	0.21 (0.08-0.52)		0.80 (0.41-1.57)	
Smoking status				
Non-current	1.00 (referent)	0.117	1.00 (referent)	0.801
Current	0.56 (0.28-1.15)		0.92 (0.49-1.75)	
Parity				
No live birth	1.00 (referent)	0.860	1.00 (referent)	0.140
≥ 1 live births	0.94 (0.47-1.87)		1.63 (0.85-3.12)	
Life time sexual partners				
≤ 5	1.00 (referent)	0.838	1.00 (referent)	0.499
> 5	1.07 (0.57-1.99)		1.22 (0.69-2.16)	
Hormonal/oral contraceptive use				
Never user	1.00 (referent)	0.791	1.00 (referent)	0.939
Ever user	1.09 (0.59-2.00)		1.02 (0.59-1.79)	
Plasma micronutrients (tertiles)				
Vitamin C (μg/mL) < 18.23, ≥18.23 - < 12.47, ≥12.47	1.43 (0.99-2.06)	0.061	0.83 (0.59-1.16)	0.271
Gamma-tocopherol (mg%) <2.36, ≥2.36 - <1.72, ≥1.72	0.61 (0.42-0.90)	0.012	0.84 (0.59-1.20)	0.333
Vitamin A (μg%) <38.4, ≥38.4 - <26.6, ≥26.6	1.22 (0.83-1.80)	0.293	1.29 (0.91-1.84)	0.160
Total carotene (μg%) <100.45, ≥100.45 - <72.61, ≥72.61	1.06 (0.72-1.57)	0.735	0.93 (0.65-1.33)	0.702
Folate and B12 combination categories				
Folate ≤19.8 ng/mL and vitamin B12 <200.6 pg/mL	1.00 (referent)		1.00 (referent)	
Folate ≤19.8 ng/mL and vitamin B12 ≥200.6 pg/mL	1.82 (0.61- 5.43)	0.285	0.93 (0.37-2.34)	0.874
Folate >19.8 ng/mL and vitamin B12 ≥200.6 pg/mL	3.92 (1.06-14.52)	0.041	1.54 (0.49-4.82)	0.458

* PBMC LINE-1 methylation ≥ 70% vs <70%

** CC LINE-1 methylation ≥ 54% vs <54%