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Research Article

Cancer Prevention Research

Folate and Vitamin B12 May Play a Critical Role in Lowering the HPV 16 Methylation–Associated Risk of Developing Higher Grades of CIN

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Abstract

We previously reported that a higher degree of methylation of CpG sites in the promoter (positions 31, 37, 43, 52, and 58) and enhancer site 7862 of human papillomavirus (HPV) 16 was associated with a lower likelihood of being diagnosed with HPV 16–associated CIN 2+. The purpose of this study was to replicate our previous findings and, in addition, to evaluate the influence of plasma concentrations of folate and vitamin B12 on the degree of HPV 16 methylation (HPV 16m). The study included 315 HPV 16-positive women diagnosed with either CIN 2+ or ≤CIN 1. Pyrosequencing technology was used to quantify the degree of HPV 16m. We reproduced the previously reported inverse association between HPV 16m and risk of being diagnosed with CIN 2+. In addition, we observed that women with higher plasma folate and HPV 16m or those with higher plasma vitamin B12 and HPV 16m were 75% ($P < 0.01$) and 60% ($P = 0.02$) less likely to be diagnosed with CIN 2+, respectively. With a tertile increase in the plasma folate or vitamin B12, there was a 50% ($P = 0.03$) and 40% ($P = 0.07$) increase in the odds of having a higher degree of HPV 16m, respectively. This study provides initial evidence that methyl donor micronutrients, folate and vitamin B12, may play an important role in maintaining a desirably high degree of methylation at specific CpG sites in the HPV *E6* promoter and enhancer that are associated with the likelihood of being diagnosed with CIN 2+. *Cancer Prev Res; 7(11); 1128–37. ©2014 AACR.*

Introduction

The oncogenic human papillomavirus (HPV) type 16 is the most frequent causative agent for developing cervical intraepithelial neoplasia (CIN) and cervical cancer worldwide. Prophylactic vaccines against HPV 16 have proved to be beneficial in lowering the development of HPV 16-associated CIN, particularly when the vaccine is received before acquiring the infection. Prophylactic vaccines are, however, unlikely to be effective in millions of women already infected with HPV 16 or in women who do not have access to vaccines for several reasons including cost, cultural barriers in accepting a vaccine, etc. Therefore, the predominant mechanisms for prevention of HPV-associated cancer for the foreseeable future will continue to be screening and treatment of CIN lesions or other non-vaccine–based approaches. Because either co-testing or primary testing for oncogenic HPV types is part of recommended cervical cancer control programs for most women (1), it is important to identify factors that might distinguish between infections that are likely to lead to neoplastic transformation and those that are self-limited.

Several *in vitro* studies have demonstrated that methylation of the HPV 16 genome results in significant repression of transcription and replication of viral DNA (2, 3). Even though the expression of HPV 16 oncogenes is essential to initiate transformation of HPV-infected epithelial cells and viral DNA methylation is likely to be involved in this process, the clinical significance of HPV 16 DNA methylation is unclear because of inconsistent findings from studies that used small sample sizes, evaluated different CpG sites of the HPV genome, and used different techniques to assess the methylation status.

The positive association of higher grades of CIN (CIN 2+) with methylation of the HPV *L1* gene appears consistent across studies (4–6) while the association with methylation in the upstream regulatory region (URR) has been less clear. The methylation status of the URR region is likely to be important, as it is located between the *L1* and *E6* genes that contain the *E6* promoter and enhancer region with

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cis-responsive elements, which regulate viral expression, replication, and packaging into viral particles (7, 8). Hublarova and colleagues (9) observed higher methylation frequencies of the enhancer and promoter region of the URR in normal cytologic specimens and lower methylation in CIN 3 and cervical cancer, with a significant association between higher methylation and lower expression of *E6*. More recent studies have used bisulfite sequencing technology to determine the methylation status of CpG sites within the URR. A study by Xi and colleagues evaluated the association between a number of methylated CpG sites in the URR using bisulfite sequencing and observed a lower overall frequency of methylation of $\geq$4 of the 11 CpG sites within the URR in women with CIN 2/3 compared with women without such lesions. The likelihood of being diagnosed with CIN 2/3 was inversely related to having four or more methylated CpGs in the URR region, regardless of covariates such as age, race, and HPV 16 variant (10).

After adjusting for demographic and possible risk factors for cervical cancer, we previously reported that a higher degree of methylation of CpG sites in the promoter region (positions 31, 37, 43, 52, and 58) and URR enhancer site 7862 was associated with a lower likelihood of being diagnosed with CIN 2+, and that an overall healthy eating index was associated with a higher degree of methylation in the enhancer site studied (11). The degree of methylation at CpG site 7862 is important as it overlaps an *E2*-binding site and is located in between two nucleosomes that form part of the HPV 16 origin of replication. Very low methylation of this site was observed in HPV 16 genomes derived from CasKi cervical cancer cells and cervical cancer tissues, and it was noted that DNA methylated at position 7862 would not replicate, and would be eliminated (12). CpG sites analyzed in this study, in relation to promoter and enhancers in the URR (LCR), were illustrated in the research article authored by Ghosh and colleagues (13). The HPV 16 promoter region we studied is also important as it contains two *E2*-binding sites, and if modified by methylation, could block *E2* from binding and enhance the transcription of *E6* and *E7* HPV oncogenes (14–16). The importance of differential methylation in these CpG sites of the HPV genome in relation to CIN risk highlights the significance of verifying previously published results as well as identifying dietary and lifestyle factors that may influence the methylation status of these important CpG sites. It remains largely undetermined whether dietary and lifestyle factors are able to influence the degree of methylation of the HPV 16 genome. Methyl donor micronutrients, such as folate and vitamin B12, could be particularly important determinants of HPV methylation because HPV uses the human methylation machinery for the methylation of its own DNA.

The purpose of this study was to replicate the association between the degree of methylation of CpG sites in the promoter region (positions 31, 37, 43, 52, and 58) and URR enhancer site 7862 and risk of CIN 2+, and to evaluate the influence of folate and vitamin B12, DNA methylation–related micronutrients, on the degree of methylation at the same CpG sites.

Materials and Methods

Study population

The study included 315 HPV 16 positive women diagnosed with abnormal cervical cells in clinics of the Health Departments in Jefferson County and surrounding counties in Alabama who were referred to the University of Alabama at Birmingham (UAB; Birmingham, AL) for further examination by colposcopy and biopsy. These women were enrolled in two R01 studies funded by the National Cancer Institute (Bethesda, MD; R01 CA102489 and R01 CA105448). The women were 19 to 45 years old, had no history of cervical cancer or other cancers 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 the 315 HPV 16 positive women, 90 women were diagnosed with CIN 2+ [cases, including CIN 2 ($n = 34$), CIN 3 ($n = 50$), or carcinoma *in situ* (CIS; $n = 6$)] and 225 were diagnosed with $\leq$CIN 1 [i.e., normal cervical epithelium ($n = 10$), HPV cytopathic effect (HCE; $n = 21$), reactive nuclear enlargement (RNE; $n = 29$), or CIN 1 ($n = 165$]. All women participated in an interview that assessed sociodemographic variables and lifestyle risk factors for cervical cancer. Pelvic examinations and collection of cervical cells and biopsies were carried out in accordance with 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 cervical cells were collected from the transformation zone with a cervical brush and immediately rinsed in 10 mL of PBS. Fasting blood samples were collected in EDTA containing blood collection tubes. Both cells and blood samples were kept cold after collection, and were transported to the laboratory on ice within 2 hours of collection. In the laboratory, cervical cell suspensions were centrifuged and the resulting pellets were resuspended in fresh PBS. Cervical cell aliquots used for HPV genotyping were stored in PreservCyt Solution at -20°C, whereas cervical cell aliquots used for methylation were stored at -80°C. Blood samples were processed within 2 hours of collection to isolate plasma, which was stored at -80°C until micronutrient assays were completed in 2 to 3 months.

HPV genotyping

DNA was extracted from cervical cells using the QIAamp MiniElute Media Kit (Qiagen, Inc.) by following the manufacturer's instruction for HPV genotyping. The HPV genotyping test (Linear array; Roche Diagnostics) was performed according to the manufacturer's instructions. Briefly, target DNA amplified by PCR uses the PGMY09/11 *L1* consensus primer system and includes co-amplification of a human cellular target, β-globin, as an internal control. Detection and HPV genotyping were achieved using a reverse line-blot method, and this test included probes to genotype for 37 HPV types [6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51,

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52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108]. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 were considered to be HR-HPV types and all other types were considered to be low-risk HPVs.

Bisulfite-pyrosequencing HPV 16 analysis

Methylation status of the *E6* enhancer (position 7862) and promoter sites (positions 31, 37, 43, 52, and 58) of HPV 16 (GeneBank accession no: NC_001526) was assessed using a pyrosequencing-based technique as described previously (17). The overall methylation was computed by taking the average methylation of the five CpG sites of the promoter and one CpG site of the enhancer. The degree of methylation of the promoter and enhancer was used both separately and combined in the data analysis.

Determination of plasma concentrations of micronutrients

Plasma concentrations of micronutrients were determined using protocols previously established and validated in the Molecular Epidemiology Laboratory at the UAB (18). Briefly, plasma folate was measured using the *Lactobacillus* microbiologic assay, plasma vitamin B12 using a competitive radiobinding assay (SimulTRAC-SNB; MP Biomedicals), plasma vitamin C by high-performance liquid chromatography (HPLC), and plasma total carotene by spectrophotometry.

Statistical analysis

Descriptive statistics were used to characterize 90 women diagnosed with CIN 2+ and 225 women diagnosed with ≤CIN 1. Differences in proportions of demographic (age, race, education, and waist circumference) and lifestyle factor variables (smoking status, lifetime number of sexual partners, and use of hormonal contraceptives) between cases and non-cases were tested using a two-sided χ^2 test. The differences in the median plasma concentrations of micronutrients (folate, vitamin B12, vitamin C, and total carotene) between cases and non-cases were determined using the two-sided Kruskal–Wallis test.

Similar to our previously published article (11), the association between the degree of methylation of CpG sites of the *E6* promoter region and enhancer and CIN 2+ was evaluated using average methylation status of the promoter and enhancer separately and as a combined measure. We used unconditional logistic regression models that specified a binary indicator of CIN 2+ diagnosis (yes/no) as the dependent variable and HPV 16 methylation (HPV 16m; ≥median vs. <median) as the primary predictor of interest after adjusting for age, race, education, waist circumference, smoking status, lifetime number of sexual partners, use of hormonal contraceptive, and plasma concentrations of vitamin C and total carotene. Because 46% of the women positive for HPV 16 were also positive for one or more of other oncogenic HPV types, to rule out the possibility that the association between HPV 16m and case status might be confounded by the methylation status of these other HR-HPVs, we repeated the analysis restricted to women positive for HPV 16 and negative for other HR-HPVs. To assess the association between the degree of HPV 16m and the severity of CIN lesions, we used multinomial regression models after adjusting for previously stated relevant risk factors of cervical cancer.

The differences in the percentages of women with ≤CIN 1, CIN 2, CIN 3 in tertile categories of plasma folate or vitamin B12, and HPV 16m (i.e., lowest tertile of plasma folate or vitamin B12 and lowest tertile of HPV 16m, the highest tertile of plasma folate or vitamin B12 and highest tertile of HPV 16m, and intermediary combinations other than extreme categories of tertiles of folate or vitamin B12 and HPV 16m) were tested using the Pearson χ^2 test. We also examined a potential interaction between plasma folate and HPV 16m (average methylation of the promoter and enhancer combined) and risk of being diagnosed with CIN 2+ after adjusting for other risk factors. The joint effects of the plasma folate and HPV 16m were estimated for combinations created using the median as the cutoff point for both variables: (i) plasma folate <14.29 ng/mL and HPV 16m <11% (used as the referent category), (ii) plasma folate <14.29 ng/mL and HPV 16m ≥11%, (iii) plasma folate ≥14.29 ng/mL and HPV 16m <11%, and (iv) plasma folate ≥14.29 ng/mL and HPV 16m ≥11%. Similarly, the interaction between plasma vitamin B12 and HPV 16m and risk of being diagnosed with CIN 2+ were tested after adjusting for relevant risk factors of cervical cancer. The joint effects of plasma vitamin B12 and HPV 16m were estimated for the following combinations: (i) plasma vitamin B12 <406.58 pg/mL and HPV 16m <11% (the referent category), (ii) plasma vitamin B12 <406.58 ng/mL and HPV 16m ≥11%, (iii) plasma vitamin B12 ≥406.58 ng/mL and HPV 16m <11%, and (iv) plasma vitamin B12 ≥406.58 ng/mL and HPV 16m ≥11%. The models testing the joint effects were adjusted for other risk factors. Because of the study size, in the above analysis, we collapsed the categories from tertiles to above and below the median for all interacting variables.

The determinants of the degree of HPV 16m (average methylation of the promoter and enhancer combined) were analyzed among non-cases. In this analysis, the exclusion of cases was necessary to avoid the possibility of reverse causation (i.e., CIN 2+ status influencing the methylation of HPV 16). We assessed the distribution of the characteristics of non-cases with a lower degree of HPV 16m and a higher degree of HPV 16m using univariate analysis. Unconditional logistic regression models were used to determine the association between age, race, education, waist circumference, smoking status, use of hormonal contraceptive and plasma concentrations of methyl micronutrients (tertiles) and the degree of HPV 16m (≥median vs. <median).

Results

Twenty-nine percent of the women infected with HPV 16 had CIN 2+ (cases), whereas 71% were free from CIN 2+ (≤CIN 1 or non-cases). Cases were more likely to be smokers compared with non-cases and this difference was of

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Table 1. Demographic, lifestyle factors, plasma concentrations of micronutrients, and the degree of HPV 16m by case status ($N = 315$)

Risk factors		Case ($n = 90$)	Non-case ($n = 225$)	*P*
Age, y	<23	39 (43%)	102 (45%)	0.75
	≥23	51 (57%)	123 (55%)	
Race	Caucasian American	57 (63%)	129 (57%)	0.33
	African American	33 (37%)	96 (43%)	
Education	High school education or higher	66 (73%)	165 (75%)	0.71
	Less than high school education	24 (27%)	54 (25%)	
Waist circumference, cm	≤88	54 (60%)	117 (54%)	0.33
	>88	36 (40%)	100 (46%)	
Smoking status	Never smoker	27 (30%)	93 (42%)	0.05
	Ever smoker	63 (70%)	130 (58%)	
Lifetime number of sexual partners	≤5	44 (52%)	97 (46%)	0.30
	>5	40 (48%)	115 (54%)	
Use of oral/hormone contraceptive	Non-user	12 (13%)	34 (16%)	0.61
	User	78 (87%)	184 (84%)	
Folate, ng/mL	<14.29	50 (56%)	102 (47%)	0.17
	≥14.29	39 (44%)	113 (53%)	
Vitamin B12, pg/mL	<406.58	49 (55%)	102 (48%)	0.24
	≥406.58	40 (45%)	112 (52%)	
Vitamin C, μg/mL	<12.38	39 (44%)	113 (53%)	0.17
	≥12.38	50 (56%)	102 (47%)	
Total carotene, μg%	<83.59	47 (53%)	101 (48%)	0.41
	≥83.59	42 (47%)	111 (52%)	
Median HPV 16m, %				
Promoter site		9.8	11.5	0.03
Enhancer site		8.0	12.0	<0.01
Promoter and enhancer site combined		10.6	11.6	<0.01

borderline significance ($P = 0.05$). Cases and non-cases were not significantly different with regard to demographics (age, race, education, and waist circumference), other lifestyle factors (lifetime number of sexual partners and oral/hormone contraceptive use), and plasma concentrations of micronutrients (folate, vitamin B12, vitamins C, and total carotene). Cases had significantly lower median HPV 16m at the promoter (9.8%) and enhancer (8.0%) sites separately and promoter and enhancer sites (10.6%) combined compared with non-cases (11.5%, 12.0%, and 11.6%, respectively; $P = 0.03$, 0.01, <0.01, respectively; Table 1).

Figure 1 depicts the range of variations in the degree of methylation in individual and combined CpG sites of the HPV 16 *E6* promoter and enhancer using box plots. The median and the interquartile range of the degree of methylation of the promoter CpGs were as follows: CpG31, 8% (5%–12%); CpG37, 13% (8%–18%); CpG43, 11% (7%–15%); CpG52, 12% (8%–16%); and CpG58, 11% (8%–16%). The median and interquartile range of the enhancer CpG7862 was 10% (6%–16%). We used the average degree of methylation of all promoter CpG sites, enhancer CpG site separately, and as average methylation of all CpG sites (promoter and enhancer combined) in our analyses.

In this study, we were able to independently reproduce the inverse association between degree of HPV 16m and the risk of being diagnosed with CIN 2+ that we previously reported in a smaller study of a similar group of women (11). We observed that the odds of being

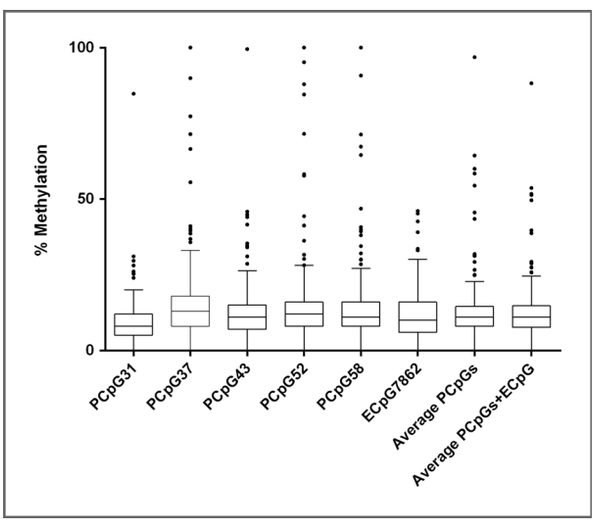

Figure 1. Box plots depicting the range of variations in the degree of methylation in individual and combined CpG sites of the HPV 16 *E6* promoter (P) and enhancer (E).

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Table 2. The association between the degree of HPV 16m and risk of being diagnosed with CIN 2+

	Cases vs. non-cases					
	Model 1[a]		Model 2[b]		Model 3[c]	
Risk factors	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Age, y						
<23	1.00	0.21	1.00	0.15	1.00	0.14
≥23	1.43 (0.82–2.54)		1.53 (0.86–2.74)		1.54 (0.87–2.75)	
Race						
Caucasian American	1.00	0.93	1.00	0.99	1.00	0.93
African American	1.03 (0.55–1.92)		1.00 (0.53–1.88)		0.97 (0.52–1.82)	
Education						
High school education or higher	1.00	0.88	1.00	0.91	1.00	0.89
Less than high school education	1.05 (0.55–1.96)		0.96 (0.50–1.81)		1.05 (0.55–1.96)	
Waist circumference, cm						
≤88	1.00	0.25	1.00	0.37	1.00	0.32
>88	0.73 (0.41–1.26)		0.78 (0.44–1.35)		0.76 (0.43–1.31)	
Smoking status						
Never smoker	1.00	0.23	1.00	0.22	1.00	0.24
Ever smoker	1.51 (0.76–3.05)		1.54 (0.77–3.12)		1.51 (0.76–3.05)	
Lifetime number of sexual partners						
≤5	1.00	0.23	1.00	0.19	1.00	0.19
>5	0.72 (0.41–1.24)		0.69 (0.39–1.20)		0.69 (0.40–1.20)	
Use of oral/hormone contraceptive						
Non-user	1.00	0.99	1.00	0.92	1.00	0.90
User	1.00 (0.47–2.22)		0.96 (0.45–2.14)		1.05 (0.49–1.14)	
Vitamin C, μg/mL						
<15.13	1.00	0.42	1.00	0.76	1.00	0.53
≥15.13	1.25 (0.73–2.13)		1.09 (0.63–1.88)		1.19 (0.69–2.04)	
Total carotene, μg%						
<87.93	1.00	0.65	1.00	0.61	1.00	0.65
≥87.93	0.88 (0.50–1.53)		0.86 (0.49–1.51)		0.88 (0.50–1.54)	
HPV 16m, %						
Promoter site						
<11	1.00	0.02	—			
≥11	0.52 (0.30–0.88)					
Enhancer site						
<10	—		1.00	<0.01	—	
≥10			0.37 (0.21–0.64)			
Promoter and enhancer site combined						
<11	—		—		1.00	<0.01
≥11					0.45 (0.26–0.78)	

[a]Model with HPV 16m of the promoter site.
[b]Model with HPV 16m of the enhancer site.
[c]Model with HPV 16m of the promoter and enhancer combined.

diagnosed with CIN 2+ were 55% lower when the degree of methylation of HPV 16 enhancer and promoter sites combined was ≥11% [OR, 0.45; 95% confidence interval (CI), 0.26–0.78; P <0.01]. In this study, a higher degree of methylation of HPV 16 promoter or enhancer sites separately was also associated with lower risk of being diagnosed with CIN 2+ (OR, 0.52; 95% CI, 0.30–0.88; P = 0.02 and OR, 0.45; 95% CI, 0.26–0.78; P <0.01; Table 2). We observed that the results remained unchanged after restricting the analysis to women positive for HPV 16 but negative for other HR-HPVs (OR, 0.47; 95% CI, 0.21–0.28; P = 0.04). In the multinomial logistic regression models adjusted for risk factors, the difference in HPV 16m was only significant between ≤CIN 1 and CIN 3 but not between ≤CIN 1 and CIN 2 or between CIN 2 and CIN 3. In this analysis, the odds

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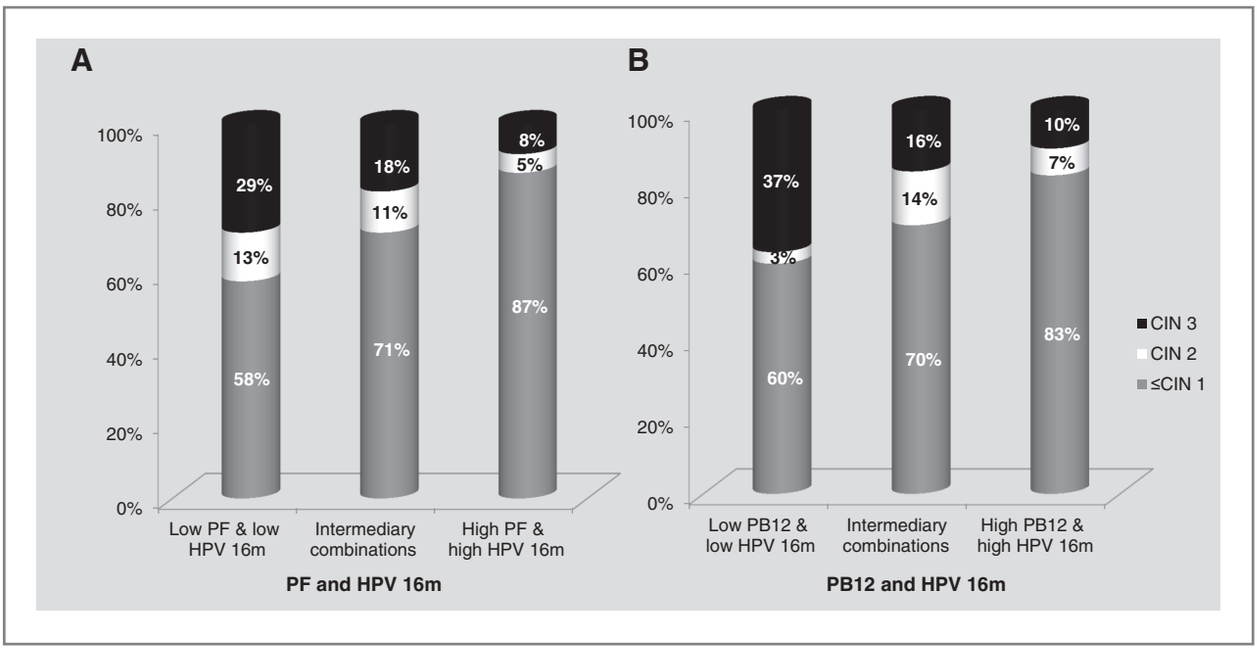

Figure 2. The percentages of women in different grades of cervical lesions by (A) plasma folate (PF) and HPV 16 methylation (HPV 16m) combinations (B) plasma vitamin B12 (PB12) and HPV 16m combinations.

of being diagnosed with ≤CIN 1 versus CIN 3 were 90% higher with every tertile increase in the degree of HPV 16m (OR, 1.90; 95% CI, 1.26–2.94; P <0.01).

The percentage distribution of women by tertiles of plasma folate or vitamin B12 and the degree of HPV 16m by the severity of CIN lesions suggested that there was a wide difference in the percentage of women diagnosed with CIN 3 in the extreme categories of plasma folate and HPV 16m combination groups (Fig. 2A), that is, women in the lowest tertile of plasma folate and the lowest tertile of HPV 16m had the highest percentage of women with CIN 3 (29%), whereas women in the highest tertile of plasma folate and highest tertile of HPV 16m had lowest percentage of women with CIN 3 (8%). Similarly, as shown in Fig. 2B, women in the lowest tertile of plasma vitamin B12 and the lowest tertile of HPV 16m had the highest percentage of women with CIN 3 (37%), whereas women in the highest tertile of plasma vitamin B12 and the highest tertile of HPV 16m had lowest percentage of women with CIN 3 (10%). As expected, the percentages of women in intermediary combinations of plasma folate or vitamin B12 and HPV 16m by the severity of CIN fell between the extreme categories, suggesting that higher concentrations of either plasma folate or B12 along with a higher degree of HPV 16m may be required to reduce the risk of CIN 3. We observed a statistically significant interaction between plasma folate and the degree of HPV 16m (Table 3). The odds of being diagnosed with CIN 2+ in women with higher plasma folate and higher HPV16m were 75% lower compared with women with lower plasma folate and lower HPV 16m (OR, 0.25; 95% CI, 0.10–0.58; P <0.01). Similarly, the odds of being diagnosed with CIN 2+ in women with higher plasma vitamin B12 and higher HPV 16m were 60% lower compared with women with lower plasma vitamin B12 and HPV 16m (OR, 0.40; 95% CI, 0.17–0.88; $P = 0.02$). Because there was only a weak correlation between folate and B12 ($r = 0.12$; $P = 0.035$), the similarity in the results with regard to folate or vitamin B12 and HPV 16m interaction on the risk of being diagnosed with CIN 2+, as reported in Table 3, is most likely to be independent of each other. In fact, the results remain unchanged when we added folate or vitamin B12 in the regression models confirming that the results observed are not due to a correlation between folate and vitamin B12.

We then analyzed the determinants of HPV 16m among non-cases. We categorized the non-cases by the median of HPV 16m (11.5%). Univariate analyses indicated that women ≥23 years old were more likely to have a higher degree of HPV 16m compared with <23 years old women, but the difference was only of borderline significance ($P = 0.08$). None of the other demographic and lifestyle factors were significantly different by HPV 16m status. Women with higher than median HPV 16m had higher median plasma concentrations of folate (16.17 ng/mL) compared with women with lower than median HPV 16m (13.46 ng/mL; $P = 0.03$). We also observed that median plasma concentrations of vitamin B12 were higher in women with higher than the median degree of HPV 16m (458.73 pg/mL) compared with women with lower than the median degree of HPV 16m (387.23 pg/mL), and this difference approached statistical significance ($P = 0.05$; Table 4).

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Table 3. Interaction between plasma folate or vitamin B12 and HPV 16m[a] and risk of being diagnosed with CIN 2+

	Cases vs. non-cases	
Plasma folate (ng/mL) or vitamin B12 (pg/mL) and HPV 16m (%) combination	**OR (95% CI)[b]**	***P***
Plasma folate (ng/mL) and HPV 16m (%) combination		
Plasma folate <14.29 ng/mL and HPV 16m <11%	1.00	
Plasma folate ≥14.29 ng/mL and HPV 16m <11%	1.10 (0.54–2.22)	0.79
Plasma folate <14.29 ng/mL and HPV 16m ≥11%	0.72 (0.35–1.46)	0.36
Plasma folate ≥14.29 ng/mL and HPV 16m ≥11%	0.25 (0.10–0.58)	<0.01
$P_{interaction}$	0.04	
Plasma B12 (pg/mL) and HPV 16m (%) combination		
Plasma vitamin B12 <406.58 pg/mL and HPV 16m <11%	1.00	
Plasma vitamin B12 ≥406.58 pg/mL and HPV 16m <11%	1.68 (0.80–3.55)	0.17
Plasma vitamin B12 <406.58 pg/mL and HPV 16m ≥11%	0.77 (0.37–1.61)	0.51
Plasma vitamin B12 ≥406.58 pg/mL and HPV 16m ≥11%	0.40 (0.17–0.88)	0.02
$P_{interaction}$	0.04	

[a]Average methylation of the promoter and enhancer.

[b]Adjusted for age, race, education, waist circumference, smoking status, use of hormonal contraceptive, lifetime number of sexual partners, and circulating concentrations of vitamin C and total carotene.

In the logistic regression models testing potential predictors of the degree of HPV 16m, we observed that with every tertile increase in the plasma concentration of folate, there was a 50% increase in the odds of having a higher degree of HPV 16m (OR, 1.51; 95% CI, 1.03–2.23; $P = 0.03$). A similar result was observed with vitamin B12, that is, for every tertile increase in the plasma concentrations of vitamin B12, there was a 40% increase in the odds of having a higher degree of HPV 16m (OR, 1.40; 95% CI, 0.97–2.04; $P = 0.07$; Table 5).

Discussion

We previously reported that a higher degree of methylation of the promoter and the enhancer region of HPV 16 was independently associated with lower risk of being diagnosed with CIN 2+. This study confirms the observation using a larger sample size. We observed that the results remained unchanged after restricting the analysis to women positive for HPV 16 but negative for other HR-HPV, suggesting that the association observed between HPV 16m and CIN 2+ cannot be explained by the degree of methylation of other HR-HPVs. Mazumder and colleagues (19) demonstrated that methylation of the HPV 16 URR gradually decreased from CIN to cervical cancer. However, their methylation assays were not quantitative and the group of CINs used was heterogeneous making it difficult to interpret their findings. In this study, using a quantitative pyrosequencing technique and well-characterized CIN groups, we demonstrated that the odds of being diagnosed with ≤CIN 1 versus CIN 3 increased 90% with each tertile increase in the degree of HPV 16 promoter and enhancer methylation after adjusting for other risk factors. Although this is the first study to suggest an independent effect of HPV 16m on the severity of CIN lesions, these results need to be confirmed in prospective studies.

Because there is no cure for HR-HPV infections, including HPV 16, control of infections by means that inhibit progression would offer a cost-effective long-term strategy to reduce the cervical cancer burden. Because HR-HPVs are carcinogenic, it is biologically plausible that micronutrients with anticarcinogenic properties exert beneficial effects against the progression of HPV infection to CIN 2+ or cervical cancer. We have previously reported that HPV 16 positive women with lower plasma concentrations of folate were nine times more likely to be diagnosed with CIN 2+ compared with HPV 16 negative women with higher folate, suggesting that women with lower folate status may have hypomethylated HPV 16, and thus may be unable to keep *E6* expression at level sufficiently low to avoid progression. This mechanism may have increased the likelihood of being diagnosed with CIN 2+. In this study, we observed that women with higher plasma concentrations of folate (≥14.29 ng/mL) and a higher degree of HPV 16m (≥11%) were 75% less likely to be diagnosed with CIN 2+, suggesting that such folate levels may allow them to keep the expression of *E6* at a lower level. Identification of folate levels that exert positive effects on HPV 16m and resulting expression of *E6* may be instrumental for designing evidence-based preventive measures using folate in the control of HPV 16-associated CIN and cervical cancer. This is a timely question to answer because the fortification of grain products with folic acid, which is mandated in the United States, has induced a population-wide increase in folic acid intake and has resulted in a wide range of folate status. According to our results, only a portion of our

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Table 4. Demographics, lifestyle factors, and plasma concentrations of folate and vitamin B12 by the degree of HPV 16m among non-cases

Risk factors		HPV 16m (%)		
		<11.5	≥11.5	*P*
Age, y	<23	56 (51%)	46 (40%)	0.08
	≥23	53 (49%)	70 (60%)	
Race	Caucasian American	66 (61%)	63 (54%)	0.34
	African American	43 (39%)	53 (46%)	
Education	High school education or higher	80 (75%)	85 (75%)	0.97
	Less than high school education	26 (25%)	28 (25%)	
Waist circumference, cm	≤88	55 (53%)	62 (55%)	0.77
	>88	49 (47%)	51 (45%)	
Smoking status	Never smokers	41 (38%)	52 (46%)	0.23
	Ever smokers	68 (62%)	62 (54%)	
Use of hormonal contraceptives	Non-user	14 (13%)	20 (18%)	0.37
	User	91 (87%)	93 (82%)	
Median plasma folate, ng/mL		13.46	16.17	0.02
Median plasma vitamin B12, pg/mL		387.23	458.73	0.05

population enrolled in the United States post-folic acid fortification era is exposed to folate levels adequate to achieve a CIN 2+ protective degree of HPV 16m. In addition to folate, we also observed that women with higher plasma concentrations of vitamin B12 and a higher degree of HPV 16m were 60% less likely to be diagnosed with CIN 2+, suggesting the importance of this methyl donor micronutrient for lowering the risk of CIN via HPV methylation. We have previously reported that women with higher plasma folate were significantly less likely to be diagnosed with CIN 2+, especially when vitamin B12 is sufficient, suggesting the significance of vitamin B12 in the process of cervical carcinogenesis (20).

Our results suggest that women infected with carcinogenic HPV types who have high plasma concentrations of folate or vitamin B 12, and achieve a high degree of CpG methylation of the *E6* promoter and enhancer are less likely to be diagnosed with CIN 2+. Factors that influence the degree of methylation in those CpG sites may reduce the risk of developing CIN 2+ in women at risk for developing such lesions. Among HPV 16 positive women not diagnosed with CIN 2+, we observed that one tertile increase in plasma folate concentrations was associated

Table 5. The associations among demographic, lifestyle factors, and plasma concentrations of folate and vitamin B12 and the degree of HPV 16m in non-cases

Risk factors		HPV 16m ≥11.5% vs. <11.5%	
		OR (95% CI)	*P*
Age, y	≥23	1.00	0.23
	<23	1.44 (0.80–2.62)	
Race	Caucasian American	1.00	0.23
	African American	1.57 (0.76–3.23)	
Educational status	High school education or higher	1.00	0.27
	Less than high school education	1.48 (0.74–3.00)	
Waist circumference, cm	≤88	1.00	0.87
	>88	0.95 (0.52–1.74)	
Smoking status	Never smokers	1.00	0.74
	Ever smokers	0.89 (0.44–1.79)	
Hormonal contraceptive	Non-users	1.00	0.13
	Users	0.53 (0.23–1.19)	
Plasma folate, ng/mL	<11.12, ≥11.12–<18.24, ≥18.24	1.51 (1.03–2.23)	0.03
Plasma vitamin B12, pg/mL	<340.56, ≥340.56–<485.21, ≥485.21	1.40 (0.97–2.04)	0.07

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with a 1.5-fold increase in HPV 16m, suggesting that improvements in folate status are beneficial for achieving CIN 2+ protective levels of HPV methylation. Similar effects were observed with vitamin B12, suggesting that improving vitamin B12 status along with folate status may also be important to reduce the HPV hypomethylation associated risk of developing CIN 2+.

Our results suggest that lower CpG methylation in the presence of lower plasma concentrations of folate and vitamin B12 influence the biology of HPV 16 and the likelihood of being diagnosed with higher grades of CIN. Evaluation of methylation in these CpG positions of the HPV 16 genome and assessment of folate/vitamin B12 status may be useful for identifying women who are at high risk of developing higher grade cervical lesions. Stronger evidence to corroborate this hypothesis should be gathered in longitudinal studies, such as prospective observational studies of HPV 16 infected women who are diagnosed with ≤CIN 1 and are characterized for folate and vitamin B12 status and HPV methylation status at baseline and followed up in time to ascertain rates of progression to CIN 2+; or better yet, in folate/vitamin B12 supplementation trials in the same groups of women. The methods we have used in this study allow evaluating DNA methylation in exfoliated cervical cells and folate and vitamin B12 in blood samples and can be easily applied to longitudinal studies in clinical settings.

In conclusion, this study provides initial evidence that two of the most important methyl donor micronutrients, folate and vitamin B12, may play an important role in maintaining a desirably high degree of methylation at specific CpG sites in the HPV *E6* promoter and enhancer that are associated with the likelihood of being diagnosed with higher grades of CIN.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Piyathilake, M.M. Chambers, N.R. Siddiqui, W.C. Bell, J.C. Edberg

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