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Folate Status and Aberrant DNA Methylation Are Associated With HPV Infection and Cervical Pathogenesis

Janet E. Flatley,[1] Kristelle McNeir,[1] Latha Balasubramani,[1] John Tidy,[3] Emma L. Stuart,[1] Tracey A. Young,[2] and Hilary J. Powers[1]

[1]Human Nutrition Unit, Faculty of Medicine, and [2]ScHARR, University of Sheffield, and [3]Sheffield Gynaecologic Cancer Centre, Royal Hallamshire Hospital, Sheffield, United Kingdom

Abstract

Aberrant DNA methylation is a recognized feature of human cancers, and folate is directly involved in DNA methylation via one-carbon metabolism. Previous reports also suggest that folate status is associated with the natural history of human papillomavirus (HPV) infection. A cross-sectional study was conducted to test the hypothesis that folate status and aberrant DNA methylation show a progressive change across stages of cervical pathology from normal cells to cervical cancer. Additionally, we postulated that a gene-specific hypermethylation profile might be used as a predictive biomarker of cervical cancer risk. DNA hypermethylation of seven tumor suppressor genes, global DNA hypomethylation, systemic folate status, and HPV status were measured in 308 women with a diagnosis of normal cervix (n = 58), low-grade cervical intraepithelial neoplasia (CIN1; n = 68), high-grade cervical intraepithelial neoplasia (CIN2, n = 56; and CIN3, n = 76), or invasive cervical cancer (ICC; n = 50). Lower folate status was associated with high-risk HPV infection (P = 0.031) and with a diagnosis of cervical intraepithelial neoplasia or invasive cervical cancer (P < 0.05). Global DNA hypomethylation was greater in women with invasive cervical cancer than all other groups (P < 0.05). A cluster of three tumor suppressor genes, *CDH1, DAPK*, and *HIC1*, displayed a significantly increased frequency of promoter methylation with progressively more severe cervical neoplasia (P < 0.05). These findings are compatible with a role for folate in modulating the risk of cervical cancer, possibly through an influence over high-risk HPV infection. *DAPK*, *CDH1*, and *HIC1* genes are potential biomarkers of cervical cancer risk. (Cancer Epidemiol Biomarkers Prev 2009;18(10):OF1–8)

Introduction

Cervical cancer is the second most common cancer in women worldwide; around half a million cases are diagnosed and >200,000 deaths are attributed to the disease annually (1). The incidence of cervical cancer is most common in developing countries, where routine cervical screening programs are largely absent.

Invasive cervical cancer is preceded by cervical intraepithelial neoplastic (CIN) lesions that may become increasingly more severe and progress towards cancer. Infection with the human papillomavirus (HPV) is the established major risk factor for cervical cancer (2). Although HPV infection is common, only a small proportion of HPV infections persist and go on to promote the development of invasive cervical cancer. Other factors must influence the susceptibility to infection with HPV and the development of HPV-induced neoplastic changes to invasive cervical cancer.

Cancer-specific changes in DNA methylation have been recognized in many cancer types. Although it is difficult to establish whether such epigenetic alterations are causative or consequential of cancer, there is evidence that they can occur early in the neoplastic process (3). There have been studies to explore the promoter methylation status of selected tumor suppressor genes in cases of cervical cancer and precancerous lesions (4-6). In general, however, these studies did not have sufficient power to detect differences in individual genes. More recently, a relatively large study, which examined changes in DNA methylation profile, from normal cervix through precancerous neoplastic lesions to invasive cervical cancer, indicated that a small panel of tumor suppressor genes may hold promise as prognostic biomarkers for cervical cancer risk (7).

In addition to promoter-site hypermethylation, a decrease in global DNA methylation is also a common feature of carcinogenesis (8). A genome-wide decrease in methylated cytosines is associated with DNA instability and proto-oncogene activation. Findings from two small studies have suggested that genomic hypomethylation may increase with increasing severity of neoplasia towards cancer (9, 10).

Adequate folate status is thought to be an important determinant of normal DNA methylation status, through provision of methyl groups by 5-methyltetrahydrofolate (11, 12). There is a growing body of epidemiologic evidence that suggests folate deficiency contributes to cancer risk at several sites (13), but findings in studies of folate and cervical cancer risk have been inconsistent. Case-control studies that estimated dietary folate intake have generally not been supportive of a protective role for folate (14-17), whereas case-control studies that used

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Requests for reprints: Hilary Powers, Human Nutrition Unit, Faculty of Medicine, University of Sheffield, Beech Hill Road, Sheffield, S10 2RX, United Kingdom. Phone: 44-0-114-2711508; Fax: 44-0-114-271-1863. E-mail: h.j.powers@sheffield.ac.uk

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biochemical measures of folate status (18-22) were more convincing. Of four randomized controlled trials using folic acid only one showed a significantly protective effect (23-26). However, most studies examining folate and cervical cancer risk were small and failed to take account of HPV infection.

The importance of folate in DNA methylation may also be relevant to a role for folate in protecting against HPV infection. Evidence suggests that folate status might be a determinant of susceptibility to HPV infection and of the infection persisting (27, 28). We conducted a cross-sectional study to test the hypothesis that folate status and aberrant DNA methylation show a progressive change across stages of cervical pathology from normal cells to cervical cancer. Additionally, we postulated that a gene-specific hypermethylation profile might be used as a predictive biomarker of cervical cancer risk.

Materials and Methods

Sample Collection. The target sample size was based on the estimated number of women required to show a significant difference in gene promoter methylation status according to grade of cervical neoplasia; the calculation was based on published data (4, 29). An analysis of standardized differences in methylation frequency among histology groups was conducted. Considering overall methylation profile (all genes), we needed 28 women per histology group in order to detect a doubling in methylation frequency among all grades, at a significance of 5% and a certainty of 85%. The sample requirement to detect a change in methylation of an individual gene was absolutely gene-dependent. We aimed to recruit 45 women from each histology group: normal; low-grade cervical intraepithelial neoplasia (CIN-1); high-grade cervical intraepithelial neoplasia (CIN-2 and CIN-3), and invasive cervical cancer.

All clinical samples were collected from women attending the Colposcopy Clinic, Jessop Wing or the Oncology department of the Royal Hallamshire Hospital, Sheffield, from 2004 to 2007, following local or regional referral. Informed signed consent was taken in the clinic at the time of the appointment. The use of human samples was approved by South Sheffield research ethics committee. Women were only excluded from the study if their clinic records indicated a positive diagnosis of a blood-borne infection.

In total, 308 women, referred to colposcopy with a cervical smear displaying cytologic abnormalities, were recruited to this cross-sectional study, which encompassed the whole range of cervical histology, namely, normal, CIN 1, CIN 2, CIN 3, and invasive cervical cancer, as diagnosed by histologic assessment.

Exfoliated cells from a cervical smear were collected into PBS. The cervical cells were then transferred into freezing medium (90% FCS, 10% DMSO) within 2 h of collection and stored at -80°C.

Nonfasted finger-prick blood samples (0.5 mL) were collected into EDTA Microvette tubes. Blood samples were stored in a 1 in 10 dilution in 0.4% ascorbic acid for folate status determination. The remaining blood was stored whole at -20°C.

Biopsy Collection and Diagnosis. A punch biopsy sample was taken of the area of cervical dysplasia under investigation and placed in formalin for histopathologic examination. Biopsy findings were interpreted by an experienced histopathologist as normal, CIN 1, CIN 2, CIN 3, or invasive cervical cancer.

HPV Testing. Cervical swabs were collected using the DNAPap Cervical Sampler that contains a brush and 1 mL of specimen transport medium. The transport medium contained 0.05% sodium azide. Swabs were stored at -20°C. Tests for the presence of high-risk HPV (HR-HPV) were carried out by the Department of Microbiology, Tata Memorial Hospital, Mumbai, India, using the Digene Hybrid Capture II Assay (HC-2). Thirteen HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) can be detected, although not distinguished, by this test (30).

DNA Preparation. Genomic DNA was isolated from cervical cells using the QIAmp DNA mini kit (Qiagen) according to the manufacturer's instructions. DNA was quantified using the Nanodrop ND-1000.

Bisulphite Modification. Genomic DNA was modified with sodium bisulphite as previously described (31). Briefly, 1 μg of DNA was denatured with 2 mol/L NaOH followed by treatment with 1 mmol/L hydroquinone and 3.5 mol/L sodium bisulphite for 5 h at 55°C. Following purification with a Wizard DNA clean up kit (Promega), the DNA was desulphonated with 3 mol/L NaOH. DNA was recovered by ethanol precipitation and centrifugation. The DNA pellet was washed in 70% ethanol and resuspended in 20 μL TE buffer (10 mmol/L Tris - HCl pH 8, 1 mmol/L EDTA).

Methyl-Specific PCR. The promoter regions of seven tumor suppressor genes (*DAPK, GSTP1, CDH1, RARβ, MGMT, hMLH1*, and *HIC1*) were analyzed for the presence of hypermethylation. The literature on hypermethylated genes in cervical cancer is limited and even further limited in precancerous lesions. The genes of choice in this study were those for which there was evidence of a role for hypermethylation for progression of cervical intraepithelial neoplasia to cervical cancer (4, 5, 29, 32). A nested two-step methyl-specific PCR was carried out on the bisulphite-treated DNA as previously described (33). The promoter regions were first amplified using primer sets specific for bisulphate-treated DNA but that did not discriminate between bisulphite-modified cytosines. The second PCR amplification step used the first round PCR product as the template to differentiate between methylated CpG sites (unmodified by bisulphite treatment) and unmethylated CpG sites (modified by bisulphite treatment). Nested primer sets for *DAPK, GSTP1, CDH1, RARβ, MGMT, hMLH1* (33), and *HIC1* (29) were obtained from Sigma-Genosys. Hot start PCR amplification was done using JumpStart Taq DNA polymerase (Sigma) under the following conditions: 95°C for 1 min; 95°C for 30 s, an annealing temperature of 54°C to 65°C for 30 s, and 72°C for 30 s for 20 cycles in first round PCR and for 25 cycles in the second round. PCR products were run on 2% agarose gels and visualized by ethidium bromide staining.

Global DNA Methylation. The global methylation status of cervical cell DNA was determined by measuring incorporation of tritium-labeled methyl groups into genomic DNA (34). Briefly, 0.5 μg of cervical cell DNA was incubated with 2 Ci of *S*-adenosyl-L-[methyl^{3}H] methionine (Perkin Elmer), 3 units of CpG *Sss*I methyltransferase

Table 1. Characteristics of the study population

	Normal	CIN1	CIN2	CIN3	Cancer	Overall
Total *n*	58	68	56	76	50	308
Mean age, y (±)	34.8 (10.8)	35.5 (10.5)	30.6 (9.01)	32.6 (9.1)	41.1* (12.6)	34.3 (10.8)
Smoking status						
Never smoked *n*	39	29	30	40	29	167 (54.2 %)
Previously smoked *n*	4	9	6	5	6	30 (9.7 %)
Current smoker *n*	15	30	20	31	15	111 (36.0 %)

*Significantly different from CIN1, CIN2, CIN3 ($P < 0.05$).

(New England Biolabs), and 1 x buffer at 30°C for 1 h. Aliquots of the reaction mixture were absorbed onto discs of Whatman DE81 paper (Fisher Scientific) and any unincorporated radiolabel was removed by washing three times in sodium phosphate buffer (0.5 mol/L, pH 7.0), followed by two washes in 70% ethanol and a final wash in absolute ethanol. Once the filter papers were dry, the remaining incorporated radiolabel was measured by scintillation counting using Ultima Gold scintillation cocktail (Perkin Elmer). Background activity values (control with no DNA) were subtracted from the sample disintegrations per minute values. DNA methylation status is inversely related to the degree of radioactive incorporation, that is, the lower the methylation of the DNA the higher the disintegrations per minute. Incorporation of methyl^{3}H into CpG Methylated jurkat genomic DNA and 5-Aza-dc treated jurkat genomic DNA (New England Biolabs) was used as reference.

Folate Measurements. Red cell folate was measured using the Beckman Access folate kit according to the manufacturer's recommendations. The Access Folate assay is a competitive binding receptor immunoassay for the determination of total folate concentration in human serum, plasma, or RBC using the Access Immunoassay Systems. Whole blood folate was measured and RBC folate calculated using the hematocrit.

Statistical Analysis. For comparison among histology groups categorical variables were examined using the χ^2 test; continuous variables were examined using ANOVA and pos-hoc analysis where appropriate. Ordinary least squares (OLS) regression models, fitted with plausible predictive variables, were developed in order to examine predictors of HPV infection, RBC folate status, and cervical histology group. Methylation status for each gene across histology groups was examined by χ^2 test, and χ^2 test for trend; factor analysis, using principle component methods, was undertaken to identify gene clusters with similar methylation profiles across histology groups. Principle component analysis attempts to identify underlying factors that explain patterns of correlation within a set of observed variables.

Results

Women were classified as belonging to one of five groups (normal, CIN1, CIN2, CIN3, and cancer) according to biopsy-determined cervical histology. All continuous variables were approximately normally distributed; no log transformations were done on the data prior to analysis.

Demographics. The ages of the 308 women included in this study ranged from 20 to 84 years with a mean age of 34 years (Table 1). ANOVA on these data revealed a significant difference in age according to histologic group. Post-hoc analysis showed that women presenting with cervical cancer were significantly older than the women with CIN1 ($P = 0.05$), CIN2 ($P = 0.001$), or CIN3 ($P = 0.002$), but not significantly older than those women with normal cervical histology. The majority (54.2%) of the women reported that they had never smoked, 36% reported being current smokers, and 9.7% stated they no longer smoked but had done so previously. There was no significant difference in smoking status across the five histological groups.

Detection of HR-HPV. Table 2 shows the HR-HPV status of the women, at the time of sample collection, according to their cervical histology diagnosis. HR-HPV was detected in 76.5% of the study population. There was a significant difference in HPV status across the cervical histologic groups; women with CIN2 or CIN3 or cancer were more likely to be HR-HPV positive than women with CIN1 or those free from cervical neoplasia ($\chi^2 = 23.29$; $P < 0.001$).

RBC Folate. The mean red cell folate concentration for the study population was higher than the national average reported for women 685 nmol/L (±293) in the most recent Diet and Nutrition Survey for adults in the United Kingdom, although the median values are comparable (35). Cervical abnormality was associated with a fall in folate status, measured as RBC folate concentration (Table 3). Women diagnosed with CIN grades 1, 2, or 3, or cancer had a significantly lower red cell folate status than those with normal cervical histology ($P = 0.041$, 0.046, 0.026, and 0.012 respectively). Table 3 also shows red cell folate concentrations according to severity of the cervical abnormality and HR-HPV status. Median RBC folate was consistently lower in HR-HPV–positive women than in HR-HPV–negative women for all histologic groups. ANOVA revealed that RBC folate status was significantly lower in women with HR-HPV infection ($P = 0.031$) than those in whom no HR-HPV has been detected; there was no interaction between HR-HPV and histologic grade.

Table 2. HPV according to CIN status

	Normal	CIN1	CIN2	CIN3	Cancer
HPV positive, *n* (%)	40 (69)	47 (72.3)	48 (87.3)	64 (85.3)	32 (65.3)
HPV negative, *n* (%)	18 (31)	18 (27.7)	7 (12.7)	11 (14.7)	17 (34.7)
Total, *n*	58	65	55	75	49

Table 3. Red cell folate status by HPV and CIN status

		Normal	CIN1	CIN2	CIN3	Cancer	Total
Total	*n*	58	65	55	75	49	302
	Red cell folate (nmol/L)						
	Mean (±)	971.2* (608)	638.3 (390)	709.3 (412.2)	734.9 (372.6)	753.0 (384.2)	770.2 (470.2)
	Median (range)	811 (146-2,871)	589 (169-1,874)	638 (177-1,671)	655 (170-1,879)	656 (74-1,630)	660 (74-2,871)
HR-HPV positive	*n*	40	47	48	64	32	231
	Red cell folate (nmol/L)						
	Mean (±)	906.2 (597.7)	656.3 (424.3)	719.7 (409.2)	698.4 (376.5)	645.9 (330.2)	724.3 (439.0)
	Median (range)	757 (366-2,871)	511 (170-1,874)	621 (177-1,671)	616 (170-1,879)	619 (74-1,630)	602 (74-2,871)
HR-HPV negative	*n*	18	18	7	11	17	71
	Red cell folate (nmol/L)						
	Mean (±)	1,121.3 (642.2)	731.5 (338.4)	704.4 (467.4)	869.1 (301.6)	917.2 (385.5)	888.23 (455.1)
	Median (range)	1,055 (146-2,282)	735 (174-1,358)	657 (247-1,567)	794 (478-1,297)	894 (289-1,465)	792.3 (146-2,282)

*Significantly higher than all other groups, $P < 0.05$.

Data were examined for differences in folate status according to alcohol consumption and socioeconomic status. Women for whom these data were available were classified as alcohol consumers (alcohol consumed daily, weekly, monthly; $n = 100$) or infrequent (never or less than monthly; $n = 22$). A comparison using Mann Whitney *U* gave no evidence on the effect of RBC folate status ($P = 0.86$).

Folate status was also examined according to socioeconomic status. Women ($n = 250$) were classified according to an index of deprivation[4] defined using postcodes. A comparison was made among women graded as low, medium, or high socioeconomic status. No evidence for an effect of socioeconomic status was found (Man Whitney U, $P = 0.99$).

Factors associating with red cell folate were examined using an OLS model. Initial examination of the data showed that significant interactions included those between age and cervical histologic class, HR-HPV and cervical histologic class, and global hypomethylation (incorporation of tritiated *S*-adenosyl methionine into DNA) and histologic class. Therefore, allowances were made for these interactions in the initial model. Age, HR-HPV status, cervical histologic class, and the interactions between age and cervical histologic status were fitted into the final model. The model had an overall significance level of $P = 0.0005$ and an R^2 of 0.097 and was fitted to 271 observations.

OLS regression showed that folate status was significantly associated with both cervical histology and HPV status ($P = 0.006$). Women who were HPV positive had a significantly lower red cell folate status than those who were HPV negative ($P = 0.032$). Similarly, red cell folate was significantly lower at all stages of cervical abnormality than in those women with a normal cervix ($P < 0.05$). In addition, individuals who had never smoked had a significantly higher red cell folate status than those who had smoked ($P = 0.029$).

Global DNA Methylation. Analysis of global methylation status was done on a total of 222 genomic DNA samples: 35 normal, 43 CIN1, 42 CIN2, 54 CIN3, and 48 cancer samples. This reduced sample size was due to constraints on the quantity of DNA available for both hypermethylation and hypomethylation analyses. A highly significant effect of cervical histology on global DNA hypomethylation was observed (Fig. 1). Post-hoc analysis revealed that incorporation of tritiated *S*-adenosyl methionine into cervical cell DNA isolated from cancer patients was significantly greater than that in all other cervical histologic grades ($P < 0.05$). This means that women with cervical cancer have a higher degree of hypomethylation of cervical cell DNA than women without cervical cancer.

Detection of Hypermethylated Genes. Six of the seven tumor supressor genes showed statistically significant changes in promoter hypermethylation frequency across the cervical histologic grades (Table 4). When necessary, to fulfill χ^2 assumptions, methylation data for more than one histology group were merged, for single genes.

Death-Associated Protein Kinase (*DAPK*). There was a significant difference in the proportion of *DAPK* promoter methylation across the histologic groups ($\chi^2 = 37.28$; $P < 0.001$). A χ^2 test for trend showed that there was a significant increase in the proportion of women in whom promoter methylation was detected with

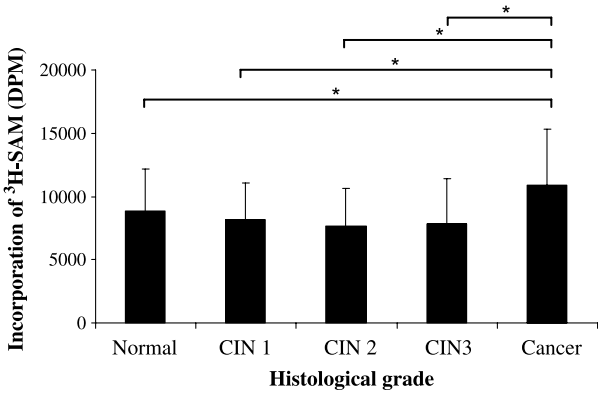

Figure 1. Global hypomethylation levels according to histologic grade. *, $P < 0.05$.

[4] British Department for Communities and Local Government (DCLG). The Indices of deprivation 2004. Available from: http://www.communities.gov.uk/archived/general-content/communities/indicesofdeprivation/216309/.

Table 4. Hypermethylation of tumor suppressor genes

Gene	Normal	CIN1	CIN2	CIN3	Cancer
DAPK	0/40 (0)	0/46 (0)	13/48 (27.1)	8/46 (17.4)	17/42 (40.5)
CDH1	1/44 (2.3)	1/55 (1.8)	5/48 (10.4)	5/54 (9.3)	8/40 (20)
HIC	0/40 (0)	4/52 (7.7)	5/47 (10.6)	3/46 (6.5)	11/45 (24.4)
MGMT	12/45 (26.7)	17/49 (34.7)	13/51 (25.5)	13/51 (25.5)	10/42 (23.8)
RARβ	3/46 (6.5)	20/47 (42.6)	3/48 (6.3)	0/51 (0)	7/44 (15.9)
hMLH1	11/51 (21.6)	15/44 (34.1)	12/50 (24)	10/49 (20.4)	6/44 (13.6)
GSTP1	3/52 (5.8)	15/56 (26.8)	8/52 (15.4)	0/49 (6.5)	0/42 (0)

NOTE: Number of samples positive for hypermethylation out of the total number of samples tested that produced a valid result (percent).

increasing cervical abnormality, with the highest proportion in the cancer group ($\chi^2 = 8.62$; $P < 0.001$).

E-Cadherin (*CDH1*). To fulfill the requirements of χ^2 analysis, normal and CIN1 classes were grouped as were CIN2, CIN3, and cancer. There was a significant difference in the frequency of methylation of the CDH1 promoter between these two groups, with the latter group having a higher frequency of methylation ($\chi^2 = 7.36$; $P = 0.007$).

Hypermethylated in Cancer (*HIC*). For χ^2 analysis, normal and CIN1 classes were grouped as were CIN2, CIN3, and cancer. There was a significant increase in the frequency of methylation in CIN 2, CIN 3, and cancer compared with CIN 1 and normal ($\chi^2 = 4.45$; $P = 0.035$).

O^6-Methylguanine-DNA Methyltransferase (*MGMT*). *MGMT* behaved similarly to *hMLH1* in showing a relatively high frequency of methylation in DNA from normal cervixes and all levels of dysplasia, but had a markedly lower methylation frequency in cancer. There was no significant difference or trend in the frequency of methylation across the histologic groups.

Retinoic Acid Receptor, Beta (*RARβ*). It was necessary to combine the CIN 2 and CIN 3 groups for χ^2 analysis. There was a significant difference in the frequency of methylation across the histologic groups ($\chi^2 = 44.04$; $P = 0.001$) with the highest rate of hypermethylation found in CIN 1 and women with cancer. There was no systematic trend observed for *RARβ*.

Human *mutL* Homologue 1(*hMLH1*). *hMLH1* showed a relatively high frequency of promoter methylation at all levels of cervical dysplasia, as well as in normal cervical cells. There was no significant difference or trend in the frequency of methylation across the histological groups.

Glutathione S-Transferase pi (*GSTP1*). CIN 2 and cancer groups were combined with CIN 3 for the purpose of χ^2 analysis. There was a significant difference in the frequency of hypermethylation across the histologic groups with the highest frequency of methylation found in women with CIN1 ($\chi^2 = 20.95$; $P < 0.001$). There was no significant trend observed across the groups.

Principle component analysis revealed two groups of genes for which there was a similar pattern of methylation across the histologic groups (Fig. 2). The first group, composed of *DAPK, CDH1*, and *HIC*, showed a significant difference in the frequency of promoter methylation ($\chi^2 = 49.19$; $P < 0.001$). A test for trend showed a significant increase in the frequency of methylation as cervical abnormality increased ($\chi^2 = 39.35$, $P < 0.001$). The second group of genes, composed of *hMLH1, RARβ*, and *GSTP1*, also showed an effect of histologic grade on promoter methylation ($P = 0.005$). In this group, the highest frequency of methylation was found in CIN1 and the lowest rate in CIN3 ($\chi^2 = 14.75$; $P = 0.005$). There was no trend (systematic increase/decrease) in methylation rate across CIN groups ($\chi^2 = 1.82$; $P = 0.177$). *MGMT* does not appear in Fig. 2 as it showed a distinct methylation profile, with a moderately high frequency of methylation across all histologic groups, but no difference between groups.

Discussion

The principal findings of this cross-sectional study of cervical neoplasia and cancer are that RBC folate status is significantly and independently associated with HR-HPV status and progression of cervical neoplasia, such that lower RBC folate concentration is more likely in women testing positive for HR-HPV infection or having abnormal cervical cytology; and three tumor suppressor genes (*DAPK, CDH1*, and *HIC1*) display an increased frequency of promoter methylation, with increasing severity of cervical neoplastic change.

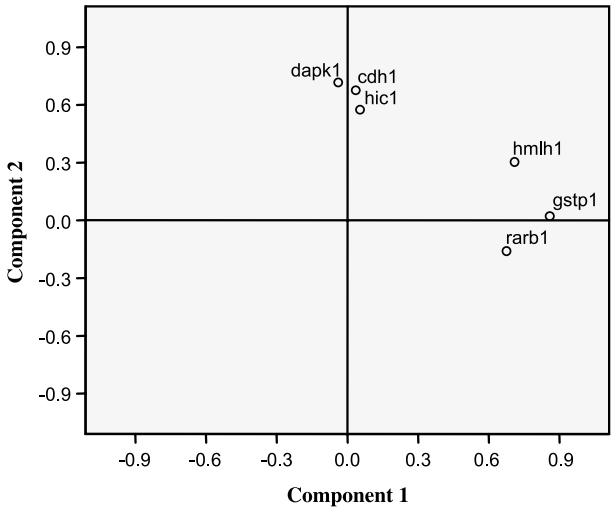

Figure 2. Component plot of gene promoter methylation showing two distinct gene clusters. Principal component analysis is a mathematical approach to reducing the amount of variability in a dataset. The principal components are artificial variables that allow us to see the similarities and differences in the methylation status in the different genes in our sample and to determine whether the genes can be grouped. In this case, the analysis identified two strong groupings: one including *DAPK, CDH*, and *HIC1*, and the other comprising *RARβ, hMLH1*, and *GSTP*.

Although there were the anticipated differences in HR-HPV status across the histologic grades, HR-HPV infection was found to be much lower than expected in women diagnosed with cancer, considering that HPV-infection has been found to be demonstrably present in 99% of women with cervical cancer (36). It is reasonable to suppose that there is a lower limit to the number of vital copies of DNA that need to be present to obtain a positive result in the Hybrid Capture II assay. We have to assume that, for technical reasons, samples in this group were more prone to false-negative results.

A lower RBC folate concentration was significantly and independently associated with a diagnosis of CIN or cancer and a positive test for HR-HPV. Regression models took into account other factors likely to confound a simple relationship among red cell folate concentration, cervical histology, and HR-HPV infection, and smoking emerged as a potentially important determinant of folate status. Women who had ever smoked had a lower RBC folate concentration than those who had never smoked. An association between smoking habits and folate status has been reported in other studies (37), and there is evidence suggesting that smoking interferes with folate metabolism (27).

Smoking is a known independent risk factor for cervical cancer in HPV-positive women (38). A pooled analysis of the International Agency for Research on Cancer multicenter case-control study found an excess risk for ever-smoking among HPV-positive women (odds ratio, 2.17; 95% confidence interval, 1.46-3.22; ref. 38). Furthermore, a recent study of >10,000 women reported an increased risk of HPV infection in current smokers compared with never smokers (39). However, smoking status was not found to have a significant association with either cervical histology or HR-HPV status in this current study population, perhaps because our sample size was insufficient to detect such associations or inaccurate self-reporting of smoking status.

A previous study reported that folate status associates with the natural history of HPV infection. In a prospective study of 537 women, Piyathilake and coworkers observed that a higher folate status was inversely associated with becoming HR-HPV positive (27). Further, upon follow-up, women with a higher folate status were significantly less likely to repeatedly test HR-HPV positive. The cohort had a mean red blood folate concentration of 894 nmol/L, reflecting a population with good folate status (40). A more recent study by the same group revealed that women with a HPV-16 infection and a lower RBC folate status had a significantly greater risk of having high-grade cervical neoplasia than women who were HPV negative with a higher RBC folate status (28).

The current study population had a mean RBC folate status comparable with the mean reported for 19- to 64-year-old women in the United Kingdom (mean, 685 nmol/L; ref. 35). Importantly, the RBC folate values reported in this study are incident observations. Women volunteered for this study prior to learning of their cervical histology or HR-HPV status and therefore had no reason to alter their diet for [cervical] health reasons prior to diagnosis. This strengthens the case for a cause-effect relationship between lower folate status and increased risk of HPV infection and cervical neoplasia. Women in many low-income countries experience diets that are very low in folate; this, in combination with the lack of screening facilities for cervical neoplasia, may exacerbate the risk of cervical cancer.

Poor folate status may contribute to cancer risk through effects on one-carbon metabolism and DNA methylation. Genome-wide hypomethylation has long been recognized as a hallmark of cancer (41). A reduction in global methylation is believed to contribute to genetic instability and activation of oncogenes (42). Hypomethylation-induced genomic instability seems also to influence the viral life-cycle and may also facilitate integration of HPV DNA into the host genome (43). Folate depletion causes a global decrease in DNA methylation in cell lines (11), and human folate deficiency has been associated with global DNA hypomethylation in lymphocytes (44) and colorectal mucosa (45). Very little is known about global DNA hypomethylation and cervical carcinogenesis. Kim et al. reported a significant association between global DNA hypomethylation and cervical histology in a small cross-sectional study of 41 subjects. Significant incremental increases in levels of hypomethylation with increasing severity of cervical neoplasia were reported (10). A second cross-sectional study by Fowler and coworkers reported a negative correlation between cervical tissue folate and serum folate concentration and the level of global hypomethylation (9). They were unable, however, to show any differences in folate status according to severity of cervical neoplasia.

In our much larger study ($n = 222$) we detected a highly significant increase in cervical cell DNA hypomethylation in cancer patients compared with all other histologic grades but no association between global DNA hypomethylation levels and RBC folate concentration. Differences in the methods between the studies may have contributed to the inconsistency in these findings. The Fowler group extracted DNA from biopsy material whereas we used exfoliated cervical cells. Although we measured cervical cell folate concentrations, we judged measurement precision to be too poor to justify presenting data in this report.

The unregulated growth of cancer cells occurs more rapidly than normal cellular growth and therefore demands more folate for DNA synthesis. Neoplastic growth may cause a shortage of folate for other processes such as DNA methylation, causing a global loss of methylation. It is plausible that a threshold level of hypomethylation is reached during the precancerous stages of growth that, once surpassed, facilitates the transformation of cells into the immortalized tumors of invasive cancer.

Promoter hypermethylation of tumor suppressor genes is another recognized hallmark of human cancer (46). Our data suggest that promoter methylation of *DAPK, CDH1*, and *HIC1* could be useful biomarkers of cervical cancer risk in women with low-grade cervical lesions.

There have been relatively few studies of promoter gene methylation in cervical neoplasia and cancer, and studies differ in terms of sample size, study design, choice of genes, and the age and race of women under study. Feng et al conducted their study in a population of 319 Senegalese women, with an average age of 44.3 years (range, 35-80 years); they characterized DNA promoter methylation of 20 candidate genes in exfoliated cervical cell samples. The study population was stratified according to five histologic diagnoses; atypical squamous cells of undetermined significance and negative diagnoses were combined as one histologic category. A

subset of genes was identified (*CDH13, DAPK, RARβ,* and *TWIST1*), in which the frequency of hypermethylation increased significantly with increasing severity of neoplasia (7). Two genes, *DAPK* and *CDH1*, displayed a similar pattern of hypermethylation according to histologic grade as in the study reported here. Although there is a great deal of heterogeneity in reports of promoter methylation in specific genes in cervical neoplasia and cancer, there is reasonable agreement across studies for these two genes. Widschwendter et al. also reported an increase in the frequency of promoter methylation in *DAPK* and *CDH1* from low-grade cervical neoplasia to cervical cancer in their small study of Austrian women (5), and Shivapurkar and colleagues reported a high frequency of promoter methylation of *DAPK* in high-grade neoplasia and cervical cancer compared with normal cervical cells (47).

In contrast to *DAPK* and *CDH1*, the reported methylation profiles for our other genes of interest vary very greatly across studies of cervical neoplasia and cancer. Wentzensen et al. (48) recently conducted a systematic literature review of gene promoter methylation in cervical tissue that highlights the highly heterogeneous nature of the data. Studies differed in some key aspects including the nature of the cervical tissue used (fresh-frozen tissue, exfoliated cells, paraffin-embedded tissue) and the methods used to determine gene methylation. Among 15 genes analyzed in detail, 7 had at least a 60% range in reported methylation frequency across studies. The study strongly suggested that differences across studies in reported methylation frequencies for the same gene in cervical neoplasia or cervical cancer may arise because of differences in target tissue, sample handling, and methylation methodology. However, *CDH1* and *DAPK* both showed mean methylation frequencies that were indicative of a progressive increase in methylation from normal tissue to invasive cervical cancer. Furthermore, *DAPK* (but not *CDH1*) showed consistent results across studies, irrespective of sample type and methylation method. The three genes, *CDH1, HIC1*, and *DAPK*, resulting from our study are all plausibly associated with cervical carcinogenesis and are reported to be methylated and silenced in cancers at various other sites (49-51).

The results of this cross-sectional study support a role for folate in modulating the risk of cervical cancer, and specifically, are compatible with a role for folate in the natural history of HPV. There was no simple relationship among folate status, DNA hypomethylation, and tumor suppressor gene hypermethylation. A relationship between lower folate status and global methylation has been described in colon systems, with a high degree of consistency (52). Furthermore, global hypomethylation can be lowered by folic acid supplementation (53), supporting a causal link. In contrast, folate is likely to be only one of many factors influencing promoter hypermethylation of tumor suppressor genes, which shows gene and tissue specificity in response to folate depletion. The understanding of the role of folate in determining risk of cervical cancer will require prospective cohort studies. A gene methylation signature in women with low-grade cervical neoplasia, which includes *DAPK, CDH1*, and *HIC1*, holds promise as a biomarker of cervical cancer risk. This too requires validation in a prospective study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Janet E. Flatley, Kristelle McNeir, Latha Balasubramani, et al.

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