Hypomethylation in Cervical Tissue: Is There a Correlation with Folate Status?\(^1\)

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Abstract

We have shown previously that DNA hypomethylation is significantly associated with grade of cervical intraepithelial neoplasia (CIN; Y. I. Kim et al., Cancer, 74: 893–899, 1994). The objective of this study was to further describe this relationship and to investigate the role of folate in the observed association of DNA hypomethylation and CIN. Eighty-three patients with abnormal PAP smear results were referred to the Cervical Dysplasia Clinic at the University of Arizona for colposcopic examination and biopsy. Patients completed a short questionnaire and provided a nonfasting serum sample. DNA hypomethylation was assessed by incubating DNA extracted from biopsy samples with \(^{[3H]}\)methyl-S-adenosylmethionine and Sss1 methylase. Cervical tissue and serum folate concentrations were assessed using a microbiological assay. All folate levels were log transformed prior to statistical analysis. The histological distribution of the samples was: 7 adjacent normal, 30 CIN I, 18 CIN II, 13 CIN III, and 11 carcinoma in situ (CIS). The mean age of participants was 29.8 ± 9.6 years. DNA hypomethylation was significantly different between select histological levels. Both cervical tissue folate and serum folate levels were significantly correlated to methyl level (P = 0.0211 and P = 0.0569, respectively). Smoking, hormonal contraceptive use, parity, and human papillomavirus infection were not associated with DNA hypomethylation or folate status. The current use of vitamins was significantly associated with serum folate level but not with methylation or cervical folate levels. These data extend our earlier findings that DNA hypomethylation is an early event in cervical carcinogenesis. To conclude that the folate level is significantly related to DNA hypomethylation, further investigation of DNA hypomethylation of specific genes is required.

Introduction

DNA methylation status is associated with carcinogenesis of many epithelial and nonepithelial cancers. DNA methylation is an early postreplicative event that is primarily found in CpG islands. SAM\(^3\) is the primary methyl donor, and folate is essential for the synthesis of SAM and the purine nucleotides. Several studies have described the association between global methylation status and carcinomas of the colon (1, 2), liver (3), intestinal type gastric (4), ovary (5), and cervix (6). Colorectal adenomas and adenocarcinomas have an average reduction of 8 and 10%, respectively, of genomic 5-methylcytosine and no significant differences between the methylation level of benign and malignant tumors (1). A graduated increase in hypomethylation was observed from normal gastric mucosa to superficial gastritis and to chronic atrophic gastritis, indicating that global DNA hypomethylation is an early event in gastric carcinogenesis (4). Global methylation levels in ovarian tumors of low malignancy potential and carcinomas were 21 and 25% lower than in cystadenomas (benign neoplasms), demonstrating that alterations in DNA methylation are early events in ovarian tumorigenesis (5). In the cervix, we showed a progressive stepwise increase of DNA hypomethylation with increasing grades of dysplasia through invasive cancer (6).

Both laboratory and clinical investigations have indicated that folate status influences carcinogenesis of several epithelial tissues. In animal studies, methyl donor deficiencies (e.g., folate, B12, and/or methionine) are associated with hepatic (3, 7, 8) and colon carcinogenesis (9). Induced methyl deficiency of laboratory animals results in DNA hypomethylation (10), structural chromosome instability (11–13), increased expression of the hypomethylated gene (14), DNA strand breaks (15), and oncogene activation (14, 16–18). Genomic hypomethylation and increased DNA methyltransferase activity in liver was noted after treatment of rodents with a methyl-deficient diet (3). However, an isolated folate-deficient diet did not induce global hypomethylation, rather hypomethylation of specific genes (15). Several clinical investigations indicate an association between folate status and risk of colorectal adenoma or cancer (19–24). Studies that examine the combined effect of high alcohol intake and low folate status find a significant risk of colorectal adenoma or cancer (25, 26) as high as a 4-fold increased risk of cancer (27).

Two reports have described a significant association between cervical dysplasia and low folate levels (28, 29). A case-control study of mild to moderate dysplasia found a 5-fold greater risk of dysplasia among women infected with HPV type 16 when RBC folate status was lower than normal (30). However, folate was not related to risk of invasive cervical cancer.

\(^1\) The abbreviations used are: SAM, S-adenosylmethionine; HPV, human papillomavirus; CIN, cervical intraepithelial; CIS, carcinoma in situ; DTT, dithiothreitol.
Hypomethylation and Folate

oral contraceptive or Depo-Provera), vitamin use, and HPV

tion and CIN. This study also sought to determine whether the
dysplasia and to determine whether serum and cervical tissue
examined cervical tissue folate levels in relation to methylation
that are known to have a high rate of spontaneous regression
early stages of cervical abnormalities. Intervention trials of
disease suggests that folate status may be more important in the
of folate status. This lack of effect of folate with invasive

-319

mM

DTTJ in a total reaction volume of 25 μl for
3 h at 37°C. The incubation mixtures were heated for 20 min at
65°C to inactivate the enzyme and were then applied to discs of
Whatman DE-81 paper (Whatman, Hillsboro, OR). The discs
were washed two times with 25 ml of 5% sodium phosphate
dibasic (pH 7.0) and then soaked in this solution for 45 min at
room temperature. The discs were subsequently dried at 95–
100°C for 30 min, and the resulting radioactivity of the DNA
retained in the discs was measured by scintillation counting using
a commercial scintillation cocktail (Econo-Safe; Re-
search Products International, Mount Prospect, IL). The amount
of radiolabel bound to a filter from an incubation mixture lacking DNA was used as a background and subtracted from the
values obtained with mixtures containing DNA. Samples lacking
DNA generally contained <1% of the label found in DNA-
containing samples. This assay quantitated the in vitro transfer
of radiolabeled methyl groups from SAM to sites in DNA that
were not methylated in vivo. Therefore, endogenous DNA
methylation status and exogenous ³Hmethyl incorporation are
inversely related.

Folate Assays. A modification of the Lactobacillus casei mi-
crobiological assay was used for the analysis of both serum and
tissue total folate levels (39). Folates were extracted from
cervical biopsy samples while on ice and protected from light.
Six hundred μl of freshly prepared 1% ascorbate (pH 6.0) was
added to the pellet, which was hand homogenized and sonicated
at 2.5% for 20 s using an ultrasonic cell disrupter. One hundred
μl of the cell homogenate were removed for the determination
of protein. The remaining 500 μl of cell homogenate were
dipped in a boiling water bath for 5 min, cooled, and then
incubated with 25 μl of Tris buffer (pH 7.2), along with 50 μl
of folate-free rat plasma conjugase preparation for 60 min at
37°C. The reaction mixture was again dipped into a boiling
water bath for 5 min, cooled, and centrifuged at 750 × g for
10 min. The supernatant was collected, filter sterilized, and stored
at −70°C until further analysis.

Folate content of the samples was then quantitatively
measured using a 96-well plate adaption of the L. casei micro-
biological assay (39). The reaction volumes containing 20 μl of
sample and folate standard (15 ng/ml) were adjusted to a total
reaction volume of 300 μl using 0.1 M phosphate buffer (pH
8.6) containing 10 mg/ml of ascorbic acid. Six serial dilutions
of sample and standard were made, resulting in folate standards
in the range of 0.005 to 0.15 μg/0.3 μl assay well. One hundred
fifty μl of L. casei medium with a concentration of 5 μl/10 μl
were added to all wells and incubated for 18 h at 37°C. The
contents of each well were then resuspended by repeated aspi-
ration and flushing with a 12-channel pipetter. Bacterial growth
was measured by reading the absorbance at 600 nm.

HPV Analysis. Exfoliated cells were collected from the cervix
and stored in standard transport medium (ViraType; Digene).

HPV status was determined using the Digene Hybrid Capture
System using an intermediate and high-risk probe that detects
HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56. The sensitivity
of this test is 1 pg of viral DNA per 100 μl of sample. The
Digene Hybrid Capture System is a rapid and relatively inex-
pensive and reliable method for detecting HPV in exfoliated
cells (40–43).

Statistical Analysis. Intercooled STATA 4.0 for Windows
3.10 statistical application was used for all statistical analyses.

All categories of continuous variables were defined by variable
distribution. Serum and tissue folate concentrations were nat-
ural log transformed to attain normal distributions for statistical
analyses. One-way ANOVA and simple linear regression were
used to test for differences in means and associations at the P ≤
Significant difference, ‘.

Methyl incorporation expressed in DPM/g DNA X 10^3.

Other selected characteristics are shown in Table 1. Mean age of participants was 29.8 ± 9.6 years. Distribution of categories total to eighty-three because of missing data.

Results

Biopsy samples of eighty-three patients were analyzed in this study with the following histologic distribution: 7 (adjacent normal), 30 (CIN I), 18 (CIN II), 13 (CIN III), and 11 (CIS). Mean age of participants was 29.8 ± 9.6 years. Distribution of other selected characteristics are shown in Table 1. Not all categories total to eighty-three because of missing data.

DNA methylation level was significantly (P < 0.05) different between select histological groups by one-way ANOVA before and after age adjustment (Fig. 1). Significant differences were observed between: (a) normal and CIN I categories; and (b) normal and CIS categories in the age-adjusted analysis. The largest methylation differences were observed in the early stage of carcinogenesis and remained stable through CIN III with a slight increase in hypomethylation observed with progression to CIS. However, the increase in hypomethylation between CIN III and CIS did not reach statistical significance.

To determine whether the association between cervical DNA hypomethylation level and grade of CIN is independent of age, hormonal contraceptive use, smoking, vitamin use, and HPV infection, we conducted an ANOVA to assess the association between these potential confounders and both cervical methylation and folate status. Only the relationship between serum folate and current vitamin use was statistically significant (P = 0.0569), although these associations were weak (r = 0.2793 and r = 0.2392, respectively). In addition, cervical tissue folate and serum folate levels were significantly associated (r = 0.4312, P < 0.001), as illustrated in Fig. 2.

The associations between serum and cervical tissue folate level and CIN are illustrated in Table 2. Neither of the folate values were significantly associated with CIN grade.

Discussion

This present study demonstrated that global DNA hypomethylation of cervical tissue is significantly different between select grades of CIN. Normal cervical tissue had the lowest level of hypomethylation, with significant increases observed in biopsy-confirmed CIN I and CIS lesions. The largest differences in methylation level occurred between normal and CIN I lesions, indicating that hypomethylation of the cervical epithelium is an early event in cervical carcinogenesis. Concentrations of folate

Fig. 1. Age-adjusted association between grade of cervical lesion and cervical DNA methylation. Normal (0.198 ± 0.07, adjusted mean ± SE (bars)), CIN I (0.402 ± 0.03), CIN II (0.369 ± 0.04), CIN III (0.358 ± 0.04), and CIS (0.485 ± 0.05). a and b: similar letters indicate significant difference at P < 0.05. Level of [3H]methyl incorporation is reciprocal to endogenous level of methylation. Therefore, increasing [3H]methyl incorporation is indicative of hypomethylation in vivo.

Table 1 Association between selected subject characteristics and level of cervical DNA methylation, cervical tissue folate concentration, and serum folate concentration

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
<th>[3H]Methyl incorporation</th>
<th>Cervical tissue folate concentration</th>
<th>Serum folate concentration</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-24</td>
<td>27 (34.6)</td>
<td>0.351 (0.16)</td>
<td>1.29 (0.54)</td>
<td>2.26 (0.72)</td>
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<tr>
<td>25-32</td>
<td>27 (34.6)</td>
<td>0.432 (0.14)</td>
<td>1.26 (0.75)</td>
<td>2.15 (0.58)</td>
</tr>
<tr>
<td>33-72</td>
<td>24 (30.8)</td>
<td>0.381 (0.16)</td>
<td>1.18 (0.91)</td>
<td>2.37 (1.05)</td>
</tr>
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<td>Parity history</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22 (28.2)</td>
<td>0.367 (0.16)</td>
<td>1.35 (0.90)</td>
<td>2.38 (0.78)</td>
</tr>
<tr>
<td>1</td>
<td>18 (23.1)</td>
<td>0.411 (0.16)</td>
<td>1.26 (0.44)</td>
<td>2.47 (0.70)</td>
</tr>
<tr>
<td>2</td>
<td>21 (26.9)</td>
<td>0.423 (0.16)</td>
<td>1.26 (0.67)</td>
<td>2.15 (0.69)</td>
</tr>
<tr>
<td>3</td>
<td>12 (15.4)</td>
<td>0.383 (0.17)</td>
<td>1.01 (0.88)</td>
<td>1.78 (0.65)</td>
</tr>
<tr>
<td>≥4</td>
<td>5 (6.4)</td>
<td>0.343 (0.07)</td>
<td>1.10 (0.93)</td>
<td>2.34 (1.24)</td>
</tr>
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<td>Hormonal contraceptive use</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>14 (25.9)</td>
<td>0.397 (0.15)</td>
<td>1.43 (0.72)</td>
<td>2.22 (1.03)</td>
</tr>
<tr>
<td>Yes</td>
<td>40 (74.1)</td>
<td>0.357 (0.17)</td>
<td>1.15 (0.71)</td>
<td>2.23 (0.71)</td>
</tr>
<tr>
<td>Duration (mo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5.0</td>
<td>12 (26.5)</td>
<td>0.360 (0.12)</td>
<td>1.17 (0.51)</td>
<td>2.50 (0.73)</td>
</tr>
<tr>
<td>5.0-24.7</td>
<td>13 (32.7)</td>
<td>0.336 (0.18)</td>
<td>1.23 (0.65)</td>
<td>1.88 (0.63)</td>
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<tr>
<td>24.8-168</td>
<td>12 (40.8)</td>
<td>0.400 (0.21)</td>
<td>1.04 (0.95)</td>
<td>2.29 (0.68)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Duration (mo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5.0</td>
<td>7 (30.4)</td>
<td>0.323 (0.18)</td>
<td>1.37 (0.88)</td>
<td>2.58 (0.60)</td>
</tr>
<tr>
<td>5.0-11.9</td>
<td>8 (34.8)</td>
<td>0.429 (0.15)</td>
<td>1.48 (0.66)</td>
<td>2.27 (0.94)</td>
</tr>
<tr>
<td>12.0-50.0</td>
<td>8 (34.8)</td>
<td>0.425 (0.15)</td>
<td>1.17 (0.35)</td>
<td>1.93 (0.89)</td>
</tr>
<tr>
<td>Vitamin use</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>48 (63.2)</td>
<td>0.390 (0.16)</td>
<td>1.13 (0.74)</td>
<td>1.94 (0.66)</td>
</tr>
<tr>
<td>Yes</td>
<td>28 (36.8)</td>
<td>0.420 (0.15)</td>
<td>1.42 (0.71)</td>
<td>2.77 (0.65)</td>
</tr>
<tr>
<td>Duration (mo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.25</td>
<td>8 (33.3)</td>
<td>0.473 (0.17)</td>
<td>1.48 (0.68)</td>
<td>2.63 (0.43)</td>
</tr>
<tr>
<td>2.25-8.0</td>
<td>8 (33.3)</td>
<td>0.367 (0.14)</td>
<td>1.38 (0.83)</td>
<td>2.57 (0.69)</td>
</tr>
<tr>
<td>8.1-12.0</td>
<td>8 (33.3)</td>
<td>0.389 (0.14)</td>
<td>1.40 (0.87)</td>
<td>3.17 (0.76)</td>
</tr>
<tr>
<td>HPV test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>27 (39.1)</td>
<td>0.392 (0.15)</td>
<td>1.40 (0.48)</td>
<td>2.30 (0.81)</td>
</tr>
<tr>
<td>Negative</td>
<td>42 (60.9)</td>
<td>0.412 (0.16)</td>
<td>1.14 (0.85)</td>
<td>2.26 (0.81)</td>
</tr>
</tbody>
</table>

* Methyl incorporation expressed in DPM/µg DNA × 10^3.
* Mean of the natural log of folate value (tissue folate, ng/mg protein; serum folate, ng/ml).
* Significant difference, P < 0.05.
Hypomethylation and Folate Status in Cervical Tissue

Fig. 2. Association between serum folate concentration and cervical tissue folate concentration. Serum and cervical tissue folate are significantly correlated ($r = 0.431; P < 0.001$).

Table 2 Association between histological grade and cervical tissue folate concentration and serum folate concentration

<table>
<thead>
<tr>
<th>Histologic grade</th>
<th>Cervical tissue folate concentration*</th>
<th>Serum folate concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>1.08 (0.81)</td>
</tr>
<tr>
<td>CIN I</td>
<td>29</td>
<td>1.16 (0.92)</td>
</tr>
<tr>
<td>CIN II</td>
<td>18</td>
<td>1.38 (0.70)</td>
</tr>
<tr>
<td>CIN III</td>
<td>13</td>
<td>1.28 (0.54)</td>
</tr>
<tr>
<td>CIS</td>
<td>11</td>
<td>1.30 (0.36)</td>
</tr>
</tbody>
</table>

* Mean of the natural log of the folate value (tissue folate, ng/mg protein; serum folate, ng/ml).

in serum and cervical tissue were weakly significantly associated with degree of cervical hypomethylation.

Global hypomethylation has been demonstrated previously to be an early event in several other epithelial cancers. Global DNA hypomethylation was present in the early stages of intestinal type gastric carcinoma (4) and in colon adenomas and adenocarcinomas (1). Global DNA hypomethylation and proliferative activity are increased in the rectal mucosa of patients with long-standing ulcerative colitis (2). Experimental folate deficiency of 0 mg/kg folate versus 8 mg/kg folate diet increases the likelihood of developing colonic neoplasia in rats treated with the carcinogen dimethylhydrazine (9). Work conducted in experimental liver carcinogenesis indicates that di"tes low in methyl donor groups (such as folate, choline, and/or methionine) increase hypomethylation (18) and increase tumor burden (7). However, moderate isolated folate deficiency (diets deplete only in folate) without the addition of a chemical carcinogen does not cause global hypomethylation of hepatic or colonic DNA or increased neoplasia in the rat model (44). However, when a chemical initiator of carcinogenesis such as dimethylhydrazine is added to the low-folate regimen, colonic carcinogenesis is induced (9). In addition, some have observed (8) hypomethylation of specific genes (such as oncogenes) after treatment with experimental diets low in methyl donor groups such as folate in the rodent model.

The progressive stepwise increase of cervical DNA hypomethylation that was demonstrated by Kim et al. (6) was not observed in the present study, although the study participants were recruited from the same clinic and are not known to have any significant lifestyle differences. The histological distribution of the two sample populations was different, and this may explain the difference in results observed from these two studies. Possibly a larger study would clarify these discrepancies. Although there appear to be differences in whether the relationship between hypomethylation and CIN is linear, both stud"ies clearly demonstrate dysplastic tissue to be hypomethylated when compared with normal tissue.

Altogether the experimental data suggest a role for folate in methylation of genomic DNA; data from our study suggest that serum and cervical tissue folate levels are weakly associated with DNA hypomethylation, although these folate levels were not associated with CIN. The apparent association of folate and methylation status in the cervix is a novel finding. No previous study has examined the relationship between cervical tissue or serum folate levels and methylation status or CIN. These data suggest that folate status may be one of several factors that determine the level of hypomethylation. None of the other factors, many of them cervical cancer risk factors, measured in this study including hormonal contraceptive use, smoking, vitamin use, and HPV infection were found to be associated with either DNA methylation or grade of dysplasia. More research is needed to determine how folate status relates to methylation status, and what the other key regulatory factors are that contribute to hypomethylation of the cervical epithelium and progression to CIN.

Hypomethylation may play an important role in cervical carcinogenesis both at the host tissue level as well as with respect to HPV-mediated cervical transformation. Although not assessed in this study, specific hypomethylation of the HPV appears to play an important regulatory role in HPV-mediated cervical carcinogenesis. Rosi et al. (45) demonstrated that integration of HPV DNA into host cells is facilitated by hypomethylation at specific promoter sites and that DNA methylation is an important regulatory pathway in the modulation of HPV expression. Regulation of viral transcription after incorporation of HPV-16 has been demonstrated previously to be suppressed by methylation of specific regulatory regions that inhibit the binding of the nuclear factor, methylation-sensitive papillomavirus transcription factor (46). The specific integration site of HPV-18 DNA sequences was demonstrated to occur near proto-oncogenes and either at or in close proximity to fragile sites (47).

No previous study has examined the relationship between serum and cervical tissue folate levels. In an experimental animal model of colon cancer, plasma and colonic mucosal folate levels were found to be highly significantly correlated ($r = 0.54$; Ref. 48). Because of the difficulty in attaining target tissue samples in epidemiological studies, the association between circulating folate concentrations and tissue concentrations has been difficult to ascertain. Our results support the existence of a significant correlation between serum and cervical tissue folate levels, indicating that serum folate status may be an adequate marker of cervical tissue folate status.

In the present study, intermediate/high-risk HPV positivity was not related to progression of dysplasia or methylation level.
The study population, however, is a select group of women who have been referred for previous abnormal findings prior to biopsy collection; therefore, the overall distribution of HPV positivity in the sample is skewed. The present study examined only global methylation level and did not examine specific genes that might have been indicative of a specific relationship between HPV positivity and cervical dysplasia. Future studies need to assess both global cervical hypomethylation and methylation patterns of specific genes.

In conclusion, cervical tissue DNA hypomethylation is significantly different between select histological categories, with the largest increase in hypomethylation occurring from the progression of normal to CIN I grade lesions. Cervical DNA hypomethylation is weakly related to both cervical tissue and serum folate levels. Cervical tissue folate concentration and serum folate concentration are linearly related, indicating that serum folate levels may be an adequate surrogate marker of tissue folate. Smoking, hormonal contraceptive use, vitamin use, parity history, and intermediate/high-risk HPV positivity were not associated with either cervical DNA methylation level or histological lesion grade.

References
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