DNA Methylation and Diet in Cancer1,2
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ABSTRACT The studies reviewed here investigate the association between folate status and DNA methylation in cancer tissues. We evaluated tissue vitamin levels and global DNA methylation, a biomarker of neoplasia, in normal lung and lung cancer tissue. Lung squamous cell carcinoma tissues exhibited global DNA hypomethylation, with decreased folate and vitamin B-12 concentrations, and increased vitamin C concentrations, relative to matched uninvolved control tissues. Breast cancer tissues also had globally hypomethylated DNA and decreased vitamin B-12 and vitamin C levels, but folate concentrations were elevated in breast cancer tissues. Global DNA methylation status in buccal mucosal cells may reflect global methylation status in lung tissues, because there was a significant association between global DNA methylation in buccal mucosal cells and malignant tissues of the lung, but not between tissue vitamin status and DNA methylation in lung tissues. We found that global DNA hypomethylation, as assessed by a radiolabeled 5-methylcytosine technique, was associated with susceptibility for development of lung cancer, which is involved in the progression of the disease. DNA methylation was also associated with the development of squamous cell carcinomas in whites but not in blacks. Overall, these studies suggest that global DNA methylation patterns may vary depending on the type of cancer, that tissue vitamin levels are associated with global DNA methylation status and that ethnicity should be considered in studies of DNA methylation. J. Nutr. 132: 3814S–3818S, 2002.

KEY WORDS: • cellular vitamins • DNA methylation • cancer susceptibility • race

Reduced dietary intake of folate has been associated with increased risk of colorectal (1,2), breast (3) and esophageal and gastric cancers (4). In addition, smokers supplemented with folate plus vitamin B-12 showed significantly greater reduction of atypical bronchial squamous metaplasia in sputum samples than for smokers in the unsupplemented placebo group (5). These studies provide an indication that folate may play a role in reducing the risk of several cancers. It is possible that folate and other “cancer-protective” vitamins exert their effect by interacting with carcinogens in tissues that are at risk for development of cancer. However, the extent to which dietary folate influences the stores of folate in cancer tissue and adjacent normal tissue has received little attention. We discuss the association between tissue vitamin status and global DNA methylation, an important and nutritionally relevant lung cancer biomarker.

Localized vitamin deficiency and cancer
Studies of vitamin deficiency at the tissue level and the relationship of this localized deficiency to risk of cancer were initiated by Dr. Carlos Krumdieck and collaborators, who performed early experiments demonstrating that hydrocarbons, including those present in tobacco smoke, were capable of inactivating folic acid and vitamin B-12 (6,7). These studies led to the proposal that carcinogenic hydrocarbons of tobacco smoke might chemically react with folates and vitamin B-12, leading to their biological inactivity (8–11). This hypothesis was tested by Piyathilake et al. (12), who reported that oral mucosal folate levels in smokers were much lower in the buccal mucosal cells of smokers than in nonsmokers. The number of micronuclei in buccal mucosal cells of smokers in this study was also higher than the number in nonsmokers, but there was no significant association between buccal mucosal folate/B-12 and the presence of micronuclei in these cells.

DNA methylation in cancer
In vertebrates, DNA is methylated by the enzyme DNA methyltransferase, which catalyzes the transfer of a methyl (m) group from S-adenosylmethionine (SAM)3 to the 5′ position of a cytosine nucleotide adjacent to guanine (CpG) to produce a 5-methyl-2′-deoxycytidine (5-mC) (Fig. 1). Methylation of cytosine residues present as CpG island sequences in DNA can repress gene transcription (13), including transcription of oncogenes and tumor suppressor genes. Two kinds of alterations in DNA methylation patterns, hypermethylation and hypomethylation.
methylation, have been observed in human tumors (13–15). In addi-
tion, both genome-wide (global) methylation and methylation asso-
ciated with specific genes (gene-specific) have been described.

Hypermethylation denotes the increased methylation of CpG-rich gene promoter sequences restricted to specific genes or genomic regions. Hypermethylation of cytosine residues present as CpG island sequences in DNA represses gene transcription (16), including transcription of tumor suppressor genes, whereas hypomethylation activates gene transcription (17,18). This unique form of regulation is thus capable of influencing progression of human cancers in the absence of mutations in oncogene or tumor suppressor genes. Rb in reti-
noblastoma (19), VHL in renal cell carcinoma (20,21), H19 in Wilms’ tumor (22) and p16 in bladder, breast, colon, lung and head and neck cancers (19,23,24) each exhibits hypermethy-
lation in cancer cells. Hypermethylation of tumor suppressor genes in cancer has been discussed in recent reviews (13,25).

In contrast to hypermethylation, DNA hypomethylation activates gene transcription (26). Global DNA hypomethylation throughout the cell genome is a common characteristic of cancer cell DNA, with the degree of hypomethylation increasing from normal through benign, primary and secondary malignancy in some types of cancer (27–29). DNA hypomethylation is apparent in the early stages of tumor progression, before obvious tumor formation (26,30,31). Global DNA hypomethylation in many cases affects the genome at large and often results in a decreased overall content of methylcytosine. It is often not clear exactly which sequences are subject to global hypomethylation. Nutritional status influences global DNA hypomethylation (see discussion later), but the influ-

TABLE 1

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Evaluate methylation and vitamin levels in same sample</td>
<td>Some day-to-day variability in assay</td>
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<tr>
<td>Can distinguish cancer cells from other cell types</td>
<td>Cannot distinguish among specific cell types</td>
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Assay of global DNA methylation

We use two methods to assess global DNA methylation. The first method involves incubation of genomic DNA with a buffered solution containing $^3$H-labeled SAM (which is the source of methyl groups in the assay) and $S_{ss}$ methylase. This incubation is followed by filtration onto Whatman DE-81 ion exchange filters, which retain the labeled DNA, and washing to remove unincorporated SAM (32). The $S_{ss}$ methylase catalyzes addition of a methyl group to all unmethylated cytosines, and hence the degree of endogenous methylation, is inversely proportional to the degree of incorporation of radio-
label into the DNA. All cells in a tissue sample (i.e., fibro-
blasts, lymphocytes and other cell types, in addition to cancer and normal epithelial cells) are evaluated with this assay. The advantage of the radiolabeled SAM assay is that the same frozen tissue specimens can be evaluated for both vitamin concentrations and global DNA methylation. Although we find that there is some variability when the same sample is assayed on different days using this method, the relative methylation values for groups of samples examined on the same day are comparable, especially when matched cancer and normal samples are used.

We also developed an immunohistochemical assay (33) that uses a monoclonal antibody specific for 5-mC (34). Using an antigen retrieval technique optimized in our laboratory (33), we found that this anti–5–mC antibody stains only the nucleus, with no cytoplasmic staining, suggesting that the antibody reaches the nucleus and has the potential to bind to methylated DNA. As with any nuclear antibody, there may be hindrance of nuclear DNA binding by endogenous DNA binding proteins in the nucleus. However, our studies to date, discussed later, indicate that nuclear staining with the anti–5–mC antibody is correlated with diagnostic tumor biomarkers, thus validating its usefulness as an indicator of events associ-
ted with tumorigenicity. Both the radiolabeled SAM incor-
poration assay and the immunohistochemical assay of global DNA methylation have advantages and disadvantages (Table 1), and a combination of both methods would likely provide a more accurate picture of global DNA hypomethylation than the use of either method alone.

In addition to the two methods described here, two new methods of assessing global DNA methylation were recently described. The cytosine extension assay (35) uses an initial di-
gestion with the methylation-sensitive restriction enzyme $H_{pa}I$, followed by a single nucleotide extension reaction using $[^3H]dCTP$, and filtration on Whatman DE-81 ion exchange filters, as described earlier for the $^3$H-SAM assay. After washing to remove unincorporated label, the filters are counted; counts in $H_{pa}I$-digested samples are subtracted from counts in undigested sample, and incorporation of $[^3H]dCTP$ is directly proportional to
the number of unmethylated (cleaved) CpG sites in the original sample. A new liquid chromatography/mass spectrometry (LC/MS) method has also been developed for the analysis of methylation of nucleotide bases in DNA (36). In this method DNA is enzymatically digested with nuclease PI, venom phosphodiesterase I and alkaline phosphatase. The four DNA bases, as well as 5-mC, in the digested mixture are separated by LC and analyzed by MS. Cytosine and 5-mC are quantified by comparison with internal and external standards, respectively. This method has a low limit of detection (100 pg), and coefficients of variance are 1.6% (within day) and 5.7% (between days).

DNA methylation and vitamin deficiency

The preliminary investigations cited here linking cellular vitamin levels with signs of neoplastic transformation did not assess concurrent expression of cancer biomarkers and cellular vitamin levels. It is important to know if localized vitamin deficiency is correlated with markers that may signal the onset of neoplasia.

We chose global DNA methylation as a biomarker of neoplasia for several reasons. First, DNA methylation is carried out via a pathway that involves folate and vitamin B-12, two of the vitamins that are of interest to us (Fig. 1). Second, deficiency of vitamins involved in methyl group transfer has been associated with the development of global DNA hypomethylation. For example, genomic DNA methylation was increased in subjects with adenomas when folate was supplemented (37). In contrast, normal human colonocytes grown without folate supplementation had decreased DNA methylation (38). Studies from our laboratory provide evidence that tissue cellular vitamin concentrations are associated with global DNA hypomethylation. We found that lung squamous cell cancer (SCC) tissues showed localized deficiencies of folate and vitamin B-12 and that deficiencies of these vitamins were associated with global DNA hypomethylation (39). In another study using paired samples of SCC and adjacent uninvolved mucosa of the lung and larynx, we reported that SCC tissues accumulated ascorbic acid and that tissue accumulation of this vitamin led to increased global DNA methylation (40). A third reason for assessing global DNA methylation as a cancer biomarker is that hypomethylation has been observed in many types of human cancer (27,41), which points to the likely significant and fundamental role played by DNA hypomethylation in tumorigenesis. The potential biological importance of global DNA hypomethylation in transformation to the neoplastic state is illustrated by the capacity of hypomethylation to induce genomic instability that leads to abnormal chromosomal structures (14,42) and to induce inactivation of oncogenes (43).

We recently evaluated vitamin levels and DNA methylation in uninvolved breast and breast cancer tissue specimens. In contrast to our observations in squamous cell (SC) lung tissues, folate concentrations were increased in breast cancer tissues in comparison to matched uninvolved control breast tissues (Table 2). However, similar to SC lung tissues, vitamin B-12 concentrations were lower, and vitamin C concentrations higher, in breast cancer than in matched uninvolved breast tissue. The same frozen tissue specimens that were assayed for vitamins were also assayed for global DNA methylation by the SAM radiolabel incorporation assay. Radiolabel incorporation was greater in the breast cancer tissue than in the normal breast tissue (i.e., breast cancer tissues were hypomethylated), with the difference approaching but not quite reaching significance, likely due to the small sample size (Table 2). We believe that the lower level of vitamin B-12 may have made a more important contribution toward DNA hypomethylation than the higher folate level in breast cancer tissue. We recently reported that lung adenocarcinomas had elevated levels of folate and vitamin C, with decreased vitamin B-12, similar to our findings for breast cancer. However, there was no difference in global DNA methylation between lung adenocarcinomas and uninvolved tissues (44) (Table 2). Differences in global DNA methylation despite a similar pattern of folate, vitamin B-12 and vitamin C in lung adenocarcinomas and breast cancer may be due to organ-specific differences in requirements for specific vitamins for global DNA methylation.

### TABLE 2

| Vitamin status and global DNA methylation of lung squamous cell carcinomas, lung adenocarcinomas and breast ductal carcinomas$^1$ |
|---|---|---|---|---|
| | Folate (ng/mg protein) | Vitamin C (ng/mg protein) | Vitamin B-12 (pg/mg protein) | Global DNA methylation (cpm/μg DNA) |
| Lung squamous cell carcinomas$^1$ | | | | |
| Matched uninvolved tissue | 2.5 | 75 | 208 | 10,508 |
| Cancer tissue | 1.7 | 441 | 109 | 35,409 |
| $P$-value | 0.04 | 0.03 | 0.03 | <0.001 |
| Lung adenocarcinomas$^2$ | | | | |
| Matched uninvolved tissue | 0.58 | 493 | 527 | 3,622 |
| Cancer tissue | 2.6 | 1,756 | 316 | 4,083 |
| $P$-value | 0.02 | 0.04 | 0.03 | 0.38 |
| Breast ductal carcinomas$^3$ | | | | |
| Matched uninvolved tissue | 0.51 | 252 | 169 | 8,698 |
| Cancer tissue | 1.48 | 1,542 | 113 | 13,665 |
| $P$-value | 0.01 | 0.02 | 0.01 | 0.06 |

$^1$ Values are medians, $n = 12$.

$^2$ Values are medians, $n = 14$.

$^3$ Values are medians, $n = 9$, except for global DNA methylation, where $n = 7$. 

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**Notes:**

- Table 2 provides a comprehensive overview of vitamin status and global DNA methylation across different types of lung and breast cancer tissues. It highlights the differences in vitamin levels and global DNA methylation between matched uninvolved and cancerous tissues.

- The table includes folate, vitamin C, and vitamin B-12 concentrations alongside global DNA methylation levels, offering a detailed comparison.

- The data suggests a potential role for vitamins in the context of DNA methylation, particularly in lung squamous cell carcinomas.

- The significance of these findings is underscored by the observed differences in vitamin levels and DNA methylation, which could be indicative of biological pathways affecting tumor development and progression.
DNA methylation in buccal mucosal cells and malignant tissues of the lung

Our earlier studies showed an association between tissue vitamin levels and DNA methylation (39,40). Many reports of associations between vitamin status and cancer risk use plasma vitamins rather than tissue vitamin concentration as the status indicator. We believe that there may be localized deficiency of vitamins in tissues at risk for neoplastic transformation and that plasma vitamin levels may not indicate these localized tissue deficiencies. Nevertheless, because tissue samples are not always easy to obtain, it is of interest to evaluate cancer markers and accompanying vitamin levels in noninvasive tissues. It is not yet clear whether vitamin levels and the level of expression of cancer biomarkers in noninvasive tissues will reflect corresponding levels in “at-risk” tissues.

We conducted a preliminary study to investigate the relationship between DNA methylation in noninvasive and easily accessible tissues, to determine whether levels of methylation in any of the noninvasive tissues would mirror methylation levels in cancer tissues. Matched samples of peripheral leukocytes, buccal mucosal cells and malignant and nonmalignant tissues of lung cancer patients (nine with primary non–small-cell and five with metastatic lung cancer) were assayed for global DNA methylation by the radiolabeled SAM acceptance assay. Patients diagnosed with primary nonsmall cell lung cancer had a radiolabeled methyl incorporation of 9,714 ± 1,259 counts per minute (cpm)/μg of DNA (mean ± SEM) in leukocytes, 25,813 ± 4,161 cpm/μg of DNA in buccal mucosal cells, and 20,359 ± 2,764 cpm/μg of DNA in malignant lung tissues. Similarly, patients diagnosed with metastatic lung cancer had 8,195 ± 909 cpm/μg of DNA in leukocytes, 29,911 ± 7,677 cpm/μg of DNA in buccal mucosal cells, and 24,677 ± 2,449 cpm/μg of DNA in malignant lung tissues. In both primary non–small cell lung cancer and metastatic lung cancer patients, methylation levels were lower in buccal mucosal cells and malignant lung tissues than in leukocytes (P < 0.05 for all comparisons). Further analysis revealed that the global DNA methylation status in buccal mucosal cells was positively associated with DNA methylation status in malignant tissues in primary non–small-cell lung cancer subjects (r = 0.70, P = 0.06, 95% CI = 0.19–1.0) but not in the metastatic lung cancer subjects (r = 0.20, P = 0.69, CI = −0.85–1.0). There was no significant association between methylation status in buccal mucosal cells and nonmalignant tissues or between leukocytes and malignant cells in either primary or in metastatic lung cancer subjects. These results suggest that global DNA methylation in buccal mucosal cells may be a good indicator of changes occurring in tissues at risk of the development of non–small cell lung cancer and may be a useful biomarker of neoplasia in this type of cancer.

Evaluation of global DNA methylation and disease susceptibility

Although DNA is known to be hypomethylated in cancer, it is not known whether this epigenetic change influences the susceptibility for cancer. There have been few studies evaluating the status of global DNA methylation in cells at various stages of carcinogenesis. We used the immunohistochemical global DNA assay to determine the relationship between DNA methylation and lung carcinogenesis (45). Lung specimens of 60 cigarette smokers who developed SCC and 30 cigarette smokers who did not were randomly selected. We found that DNA methylation scores of normal bronchial epithelial cells in noncancer specimens were not significantly different from those of uninvolved bronchial epithelial cells adjacent to SCC (P = 0.67). The DNA in epithelial hyperplastic lesions of noncancer tissues, however, had a significantly higher 5-mC staining score, compared with hyperplastic lesions adjacent to SCC (P = 0.02). Normal bronchial epithelial cell 5-mC groups in noncancer tissue specimens were more highly stained than either SCC-associated epithelial hyperplasia or SCC (P < 0.0001 and 0.002, respectively). The 5-mC scores were not significantly different between SCC-associated uninvolved bronchial epithelial cells and epithelial hyperplasia, but were different between epithelial hyperplasia and SCC, as well as between uninvolved bronchial epithelial cells and SCC (Wilcoxon sign rank test P-values 0.49, 0.01 and 0.0005, respectively). These observations suggest that altered global DNA methylation is an important epigenetic determinant of susceptibility for lung cancer.

Because breast carcinomas had been reported to have a large variation in global DNA methylation among subjects (46), in our analysis of associations between methylation and pathological features of tumors, we determined the ratio between 5-mC scores of SCC and matched uninvolved bronchial mucosa in 46 of the 60 smokers described earlier. A lower the ratio represents hypomethylation in SCC compared with adjacent uninvolved tissues. Results showed that the the ratio was significantly lower with advanced stage and size of the tumor (45). The SCC/U ratio was threefold lower in subjects diagnosed with distant metastasis, but this difference did not reach statistical significance, likely because very few of the subjects presented had distant metastasis at the time of surgery. The SCC/U ratio appeared to be unrelated to nodal status and grade of differentiation of the tumor. These results suggest that altered global DNA methylation is important in the progression of SCCs of the lung.

Effect of race and age on global DNA methylation in SCC of the lung

Very few studies have focused on the influence of race on global DNA methylation. We recently evaluated the extent to which global DNA methylation is modified by race and age and how these modifications affect the development and progression of SCCs of the lung (47). The immunohistochemical assay was used to evaluate global methylation in SCC, in associated uninvolved bronchial mucosa and in epithelial hyperplasia of 53 whites and 23 blacks. The 5-mC scores of SCC (0.59 ± 0.06) were significantly lower than those of uninvolved bronchial mucosa (UBM) (0.87 ± 0.07) and epithelial hyperplasia (EH) (0.82 ± 0.07) in whites (P < 0.05). In blacks, however, 5-mC staining of SCC (0.53 ± 0.09) was not significantly different from those of UBM (0.60 ± 0.09) or EH (0.54 ± 0.14). These results suggest that global DNA hypomethylation is associated with the development of SCCs in whites but not in blacks.

Associations of global methylation with age revealed that 5-mC scores in whites were lower in subjects 65 y of age than in subjects >65 y of age. Because cancers in younger subjects generally tend to be more aggressive than cancers in older subjects, these results could mean that hypomethylation contributes to the aggressiveness of cancers of younger whites. These initial studies suggest that the methylation status of DNA may affect the development, aggressiveness and prognosis of SCCs in whites but play a less important role in blacks. These observations suggest that careful attention should be given to racial distribution of study populations in investigations of DNA methylation (47).

In future studies, we will determine the relationship between global DNA methylation and expression of both intermediate end point biomarkers and nutrient-related biomarkers. We will also evaluate the methylation status of specific genes in relation to global DNA methylation and cellular and circulating concentrations of cancer-protective nutrients. Finally, we plan to determine whether
cellular vitamin depletion influences DNA methylation during the development of resistance to chemotherapeutic agents.

In summary, earlier studies of global DNA methylation suggested that the DNA of cancer tissues is generally hypomethylated relative to noncancer tissue. More recent studies indicate that global DNA methylation patterns may vary depending on the type of cancer, that tissue vitamin levels are associated with global DNA methylation status and that this should be considered in studies of DNA methylation.

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LITERATURE CITED


