# Epithelial Cell Folate Depletion Occurs in Neoplastic But Not Adjacent Normal Colon Mucosa

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***Background & Aims:*** **Restricted folate supply is associated with the development of carcinoma, and folate supplements have a protective effect in colorectal carcinoma. This effect may be mediated through correction of local folate deficiency. The aim of this study was to define the folate content of neoplastic colonic epithelial cells and its relation to that of adjacent normal tissue and circulating levels.** ***Methods:*** **Epithelial cells were isolated from endoscopic biopsy specimens of normal, adenocarcinoma, adenoma, and adjacent normal colonic mucosa by ion chelation. Intracellular folate levels were determined by microbiological assay.** ***Results:*** **Folate levels in carcinoma specimens were lower than in adjacent normal tissue ($P < 0.02$). Levels in adenoma epithelial cells were lower than in adjacent normal tissue, although this did not reach statistical significance ($P < 0.06$). Epithelial cells from normal tissue and mucosa adjacent to tumors and adenomata had similar folate contents. Blood folate and vitamin $B_{12}$ indices for all groups were normal.** ***Conclusions:*** **Malignant colon epithelial cells show a relative localized folate deficiency. However, there is no evidence for the occurrence of generalized mucosal folate deficiency. This finding suggests that folate supplements do not inhibit carcinogenesis through correction of localized folate depletion.**

The epidemiology of colorectal carcinoma (CRC) suggests exogenous (dietary constituents) and endogenous (bile acid metabolites) substances to influence mucosal epithelial cell proliferation and progression to malignancy. A protective role has been ascribed to vitamins and micronutrients in carcinogenesis.[1,2] Folate is of particular interest because this vitamin is required for all cellular one-carbon transfer reactions including DNA methylation and thymidine synthesis.[3] Folate supplementation favorably influences epithelial dysplasia in humans,[4,5] reduces colon tumor load in animals exposed to carcinogens,[6] and protects against the development of colonic neoplasia in patients using sulfonamide-containing drugs.[7]

The efficacy of folate in suppressing epithelial neoplasia may be related to the importance of DNA methylation in cell homeostasis. In vitro studies show that focal loss of methyl groups, which influences gene expression and generalized genomic hypomethylation, is a feature of several carcinomas.[8] Increased levels of mucosal genomic methylation after supplementation with supraphysiological doses of folate supports this hypothesis.[9] These and similar studies suggest a general theory that carcinogenesis is related to an epigenetic factor, altered patterns of genomic methylation,[10] although, they do not show a direct link between localized folate depletion and neoplasia.

Low folate levels occur in mixed cell homogenates of potentially premalignant colonic adenomatous polyps.[11] However, folate deficiency has not been shown in cells that produce CRC, the colonic epithelial cell (colonocytes). Furthermore, the relation between the folate content of colon adenoma or tumor and adjacent normal epithelial cells remains to be defined. Consequently, there is little evidence to show that folate supplementation influences carcinogenesis through the correction of tissue folate deficiency.

The aim of this study was to define the folate content of epithelial cells isolated from premalignant and malignant distal colonic lesions and to compare folate content in both adjacent normal colonocytes and tissue specimens taken from nontumor-bearing mucosa.

## Materials and Methods

### Tissue Collection

Colonic biopsy specimens were obtained at endoscopy under a protocol approved by the Ethics Committee of the Federated Voluntary Dublin Hospitals.

Patient details including medical history and current medication were recorded before endoscopy. Venous blood was drawn for the estimation of red cell folate, serum folate, and vitamin $B_{12}$ levels. Pathology in enrolled patients included distal colonic carcinoma (within 30 cm of the anal verge) (n

*Abbreviation used in this report:* CRC, colorectal carcinoma.


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= 12), distal colon adenomatous polyps (n = 7), and control patients (n = 8). Age-matched control patients had no history of colorectal neoplasia and underwent endoscopy for the investigation of altered bowel habit or rectal blood loss. They had normal endoscopic findings defined by normal macroscopic appearance and absence of inflammation on rectal biopsy specimens. All tumors were moderately differentiated adenocarcinomas and diagnosed as Dukes' disease stage B after surgery. All adenomatous polyps showed high-grade dysplasia with no evidence of coexisting frank malignancy. No patients were taking folate supplements or antifolate medication.

In patients with colonic tumors or adenomatous polyps, biopsy specimens were taken from both the lesion itself and from normal-appearing mucosa within 5 cm of the lesion (adjacent). Biopsy specimens from lesions for folate content estimation were taken before those for histopathology, and blood contamination was avoided. Biopsy specimens from control patients were taken at 30 cm from the anal verge.

## Colonic Epithelial Cell Isolation

Four endoscopic biopsy specimens from each area of interest were taken into calcium- and magnesium-free Hank's buffered saline solution (Life Technologies, Paisley, Scotland) supplemented with 0.3% bovine serum albumin, penicillin, and gentamicin containing 0.75 mmol/L dithiothreitol (Sigma, St. Louis, MO) and processed for epithelial cell isolation as previously described.[12] Briefly, biopsy specimens were allowed to stand in this solution for 3 hours at room temperature. Subsequently, the tissue was transferred to fresh medium (calcium- and magnesium-free Hank's buffered salt solution supplemented with 0.3% bovine serum albumin) containing 2 mmol/L ethylenediaminetetraacetic acid and placed on an inclined (45°), rotating table at 37°C for 1 hour. The resulting cell suspension was washed and pelleted in Hank's buffered salt solution. Cells were counted and viability was assessed using ethidium bromide–acridine orange. Cells were saved in 500 µL ascorbate 0.1% in phosphate-buffered saline (PBS) at −20°C until assayed.

## Flow Cytometry

Aliquots of epithelial cell isolates were assessed for lamina propria contamination and intraepithelial lymphocyte content by flow cytometry (FACScan; Becton Dickinson, Antwerp, Belgium) using the following monoclonal antibodies and fluorescent conjugates: Ber-Ep-4 (epithelium) (Dako, Glostrup, Denmark); CD3 (T cells) (Becton Dickinson); CD14 (monocytes) (Becton Dickinson); CD19 (B cells) (Becton Dickinson) and R155 (dendritic cells, a kind gift from Dr. A. Whelan, St. James's Hospital, Dublin, Ireland); rabbit anti-mouse fluorescein isothiocyanate (Dako); and rabbit anti-mouse phycoerythrin (Dako). Simultest immunoglobulin (Ig) G1 and IgG2a (Becton Dickinson) were used as control antibodies. Lamina propria remnants from the epithelial compartment isolation were digested in collagenase type IV 50 U/mL (Sigma) for 1 hour at 37°C and used as positive controls for the determination of leukocyte contamination.

Cells ($2.5 \times 10^6$) were suspended in fluorescence-activated cell sorter buffer (PBS, 0.1% bovine serum albumin, and 0.01% sodium azide) and washed. The cells were then incubated with previously determined optimal concentrations of the various monoclonal antibodies for 30 minutes at 4°C. After two more washes, cells were incubated similarly with a second antibody or fluorescent conjugate and again washed twice. Flow cytometry was performed using a FACScan (Becton-Dickinson), and $10 \times 10^3$ events were saved for analysis. Data were analyzed using Lysys II software (Becton Dickinson).

## Folate Assay

Cell isolates were defrosted, sonicated (Cell Disruptor B15; Branson, Utrecht, Netherlands), and deconjugated to permit microbiological assay. Three hundred microliters of sonicate was placed in a water bath at 100°C for 10 minutes. After cooling, 10 µL of chicken pancreas conjugase (a gift from Dr. A. Molloy, Trinity College, Dublin, Ireland) was added and the suspension incubated at 37°C for 2 hours. After incubation, the suspension was again boiled. Aliquots were assayed for folate content using a microtiter plate method previously described.[13] Recovery of folate was estimated by addition of tritium-labeled pteroylmonoglutamate (Amersham). A yeast extract (0.1%) control was used to ensure uniform conjugase activity between assays. Serum, red cell folate, and vitamin $B_{12}$ levels were measured by standard microbiological assay.

Folate results were standardized with respect to spectrophotometric determination of sonicate DNA content using bromodeoxyuridine. Isolate DNA content of tumor specimens correlated closely ($r = 0.85$; $P < 0.001$) with protein content (BCA protein assay; Pierce, Rockford, IL).

The ability of the microtiter assay to detect folate levels in neoplastic cells was verified by a colonic epithelial cell line cultured in varying folate concentrations.

HT29 (cl.19A) cells, a colonic epithelium carcinoma line,[14] were grown to confluency in 5 days in 25-$cm^2$ culture flasks (Costar, Cambridge, MA) in Dulbecco's modified essential medium (Life Technologies, Paisley, Scotland) supplemented with 1% nonessential amino acids (Sigma), 10% fetal calf serum, and gentamicin. Folic acid (Sigma) was added to this medium in the concentrations of 0 µmol/L, 10 µmol/L, 100 µmol/L, or 1 mmol/L (Sigma). Medium was refreshed at 24 hours and every 48 hours thereafter. The level of folate supplement did not influence rate of confluency. At day 7, medium was discarded and the cells were washed three times with PBS. Cell monolayers were disrupted with trypsin–ethylenediaminetetraacetic acid (Life Technologies). The single cell isolate was washed three times in PBS, pelleted, resuspended in 1 mL of ascorbate buffer, and saved at −20°C. After thawing, cell suspensions were sonicated and assayed for folate content. These experiments were performed in quadruplicate. Dulbecco's modified essential medium contains 2.5 µg/L folate.

## Statistical Analysis

Statistical analysis was performed using SPSS for Windows 6.0 (SPSS Inc., New York, NY). Data were compared

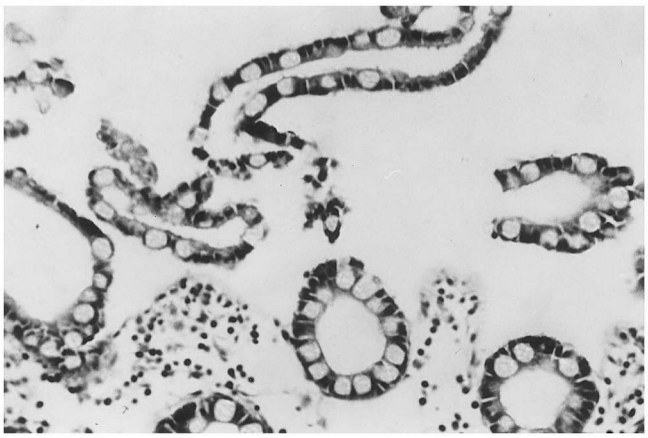

**Figure 1.** Epithelial cell isolation from endoscopic biopsy specimens. Biopsy specimens were removed from ethylenediaminetetraacetic acid solution at 45 minutes. The epithelial cell layer is free of an intact basement membrane. There is retraction of the lamina propria (original magnification 64×).

using Student's *t* test. Paired tests were used where appropriate. Correlation values are given as the Pearson correlation coefficient ($r$). Data are expressed as mean value ± SEM. $P$ values of $<0.05$ are considered significant.

## Results

Mean cell yields (viability) from adjacent normal tissue or polyps or tumors were $2.98 \pm 0.34 \times 10^6$ (83%) and $2.01 \pm 0.08 \times 10^6$ (77%), respectively. Mean cell yield from control patient biopsy specimens was $3.1 \pm 0.5 \times 10^6$ (80%). Light microscopy showed that the biopsy epithelial basement membrane was intact after ion chelation treatment (Figure 1). The proportion of intraepithelial lymphocytes present in isolates was similar between tumor (4.9% ± 0.5%) and control (4.7% ± 0.6%) groups. Lamina propria contamination of all isolates was <1% (Figure 2).

The intracellular folate content of the HT29 cells varied with medium folate content (Figure 3).

Recovery of folate was in the range of 94%–110%. The interassay and intra-assay coefficients of variation for the folate assay were <7%. Folate was undetectable in Hank's buffered salt solution supplemented with 0.3% bovine serum albumin. Cell viability has been shown previously not to influence detectable folate content.[12] The folate content of epithelial cells isolated from normal mucosa was 18.5 ± 1.56 pg/μg DNA. Cellular folate levels for adenomas and tumors (adjacent normal cell level) were 15.5 ± 2.05 pg/μg DNA (20.18 ± 2.18 pg/μg DNA; $P < 0.06$) and 15.07 ± 1.06 pg/μg DNA (18.03 ± 1.10 pg/μg DNA; $P < 0.02$), respectively (Figure 4). The differences between adjacent epithelial cell folate levels for adenomas or tumors and normal controls were not significant. A similar relation between tissue folate concentrations was seen when results were calculated in terms of isolate protein content (data not shown).

Serum folate levels for control and tumor or adenoma groups were 8.6 ± 1.1 and 6.77 ± 1.28 ng/mL, respectively ($P > 0.05$). Red cell folate concentrations for these groups were 340 ± 39 and 361 ± 72.4 ng/mL, respectively. Serum and red cell folate levels correlated ($r = 0.56$, $P < 0.01$). Neither serum nor red cell folate levels correlated with tissue levels in any group. Serum vitamin $B_{12}$ levels for all groups were within normal limits (data not shown).

## Discussion

A significant difference has been identified between the folate content of colon tumor epithelial cells and that of adjacent normal cells. Because enriched epithelial isolates were used, these results more accurately reflect the folate content of those cells that give rise to CRC than is permitted by the use of whole mucosal biopsy homogenates.[11]

Folate coenzymes are essential for cellular one-carbon transfer reactions, specifically protein and DNA methylation and nucleotide synthesis,[15] which identify a potential role in carcinogenesis.[2,16] The biological effects of folate deficiency, including defective DNA synthesis and impaired cell replication, are well recognized[17]: misincorporation of nucleotides into nascent DNA,[18] increased susceptibility to genetic damage,[19] alterations in folate pools,[20] instability of folate binding protein messenger RNA,[21] and altered gene expression caused by genomic hypomethylation contribute to this potential.[22–24] However, care must be used when extrapolating from cell culture studies to the clinical situation. The altered cell metabolism and association with carcinogenesis reported for in vitro and animal studies result from extreme folate depletion that is rarely seen in the clinical setting. Additionally, the use of hypermethylating agents (dimethylhydrazine) in animal tumorigenesis studies and a report that hypomethylation may have a suppressive effect on intestinal neoplasia[25] further complicate the issue.

The relation between the degree of folate repletion and carcinogenesis may result from very different processes operating at low and high concentrations of this vitamin. Whereas in vitro studies indicate a potential direct causative relation at very low folate levels, intervention and epidemiological studies suggest that at higher folate levels (supraphysiological) the beneficial effects of folate stem from overriding mechanisms driving neoplastic transformation other than simply deficiency.

No evidence has been found for the occurrence of very

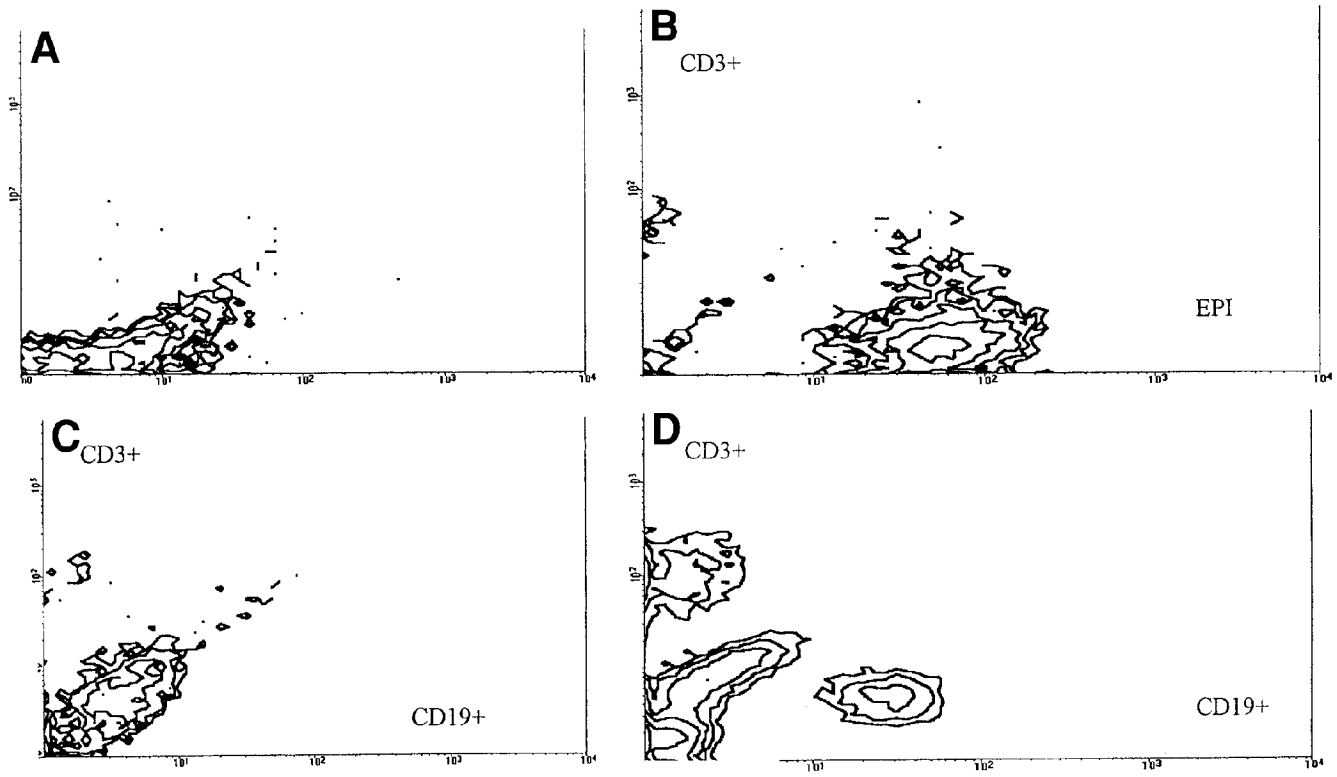


**Figure 2.** FACScan analysis of cell isolates from colonic biopsy specimens. Cells were labeled with anti-CD3 (T cells), anti-CD19 (B cells), and Ber-Ep-4 (epithelial cells). (*A*) Control cell isolate stained with IgG1 and IgG2a antibodies followed by rabbit anti-mouse phycoerythrin and rabbit anti-mouse fluorescein isothiocyanate conjugates. No difference in control staining pattern was observed between epithelial and lamina propria compartments. (*B*) Epithelial compartment isolated by ion chelation showing a predominantly epithelial cell population (x axis) with some intraepithelial lymphocytes (y axis). (*C*) Epithelial compartment is stained with anti-CD19 (x axis) and anti-CD3 (y axis) to detect lamina propria contamination. (*D*) Lamina propria compartment is isolated by collagenase digestion and labeled with anti-CD3 (x axis) and anti-CD19 (y axis).

low folate levels in colon mucosa. Indeed, although levels were lower in carcinomatous epithelium, with a downward trend in premalignant cells, they still remained within the range found in control patients. The close correlation between DNA and protein levels suggests that the low level of folate in adenoma and tumor epithelium is not artifactual and results from abnormal neoplastic cell DNA content. These findings may mirror the effect of abnormal rates of cell turnover or abnormal cellular enzyme activity, such as methylene tetrahydrofolate reductase. However, neither of these possibilities negate any potential coeffects that low folate may have on cellular metabolism, regardless of how the deficiency is induced.

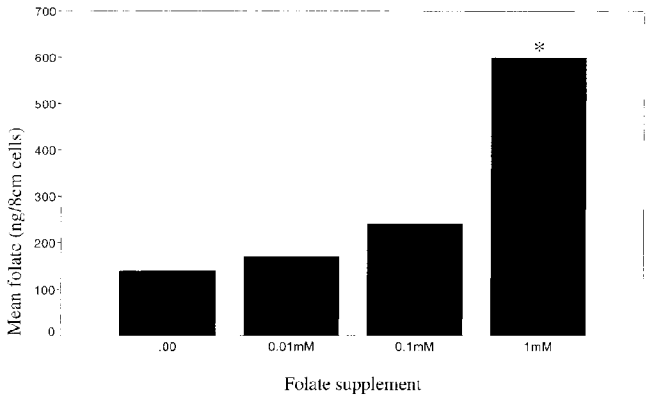


**Figure 3.** The folate content of HT29 cells grown in varying concentrations of folic acid. *Asterisk* represents 25% of actual value.

Folate availability directly influences DNA methylation[9,26]; however, moderate folate deficiency identified in this study does not result in global gene or c-myc-specific hypomethylation.[11] This reflects a capacity to redirect methyl groups in times of shortage.[27] The results from the present study suggest that the genomic hypomethylation noted in CRC does not develop from a reduced supply of folate, but it does not exclude impaired use. However, the possibility cannot be excluded that transient episodes of more profound folate deficiency may occur over the long period of time associated with the development of colonic mucosal lesions.

The requirements for folate may be higher in some tissues (digestive mucosa and cervix) and under certain conditions (neoplasia) than others, leading to localized deficiencies, despite blood levels being within the normal

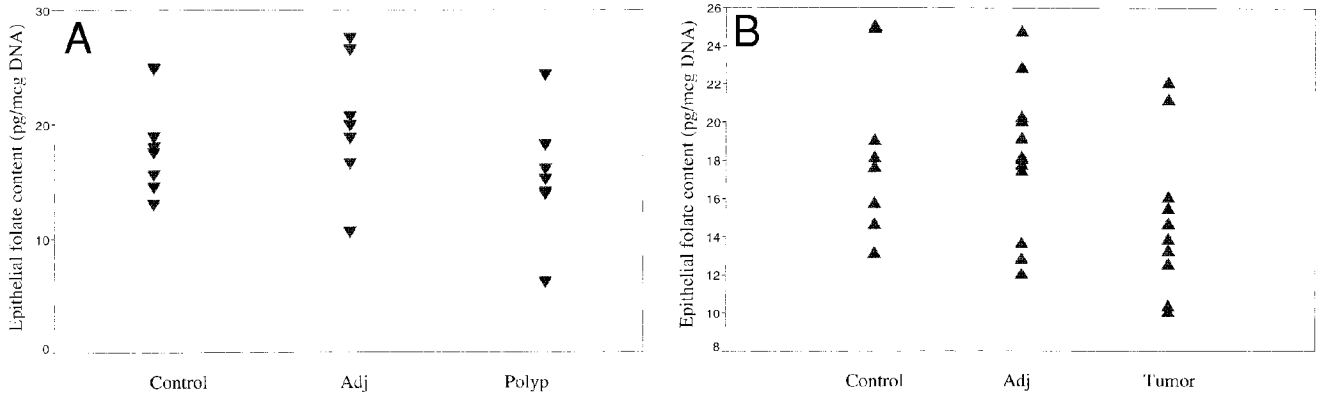


**Figure 4.** (*A*) Folate content of colonic epithelial cells isolated from normal colon (control, n = 8), colonic adenomatous polyps (polyp, n = 7), and normal-appearing tissue (adj) adjacent to the polyp. $P = 0.06$. (*B*) Folate content of colonic epithelial cells isolated from normal colon (control, n = 8), colonic adenocarcinoma (tumor, n = 12), and normal-appearing tissue (adj) adjacent to the tumor. $P < 0.02$.

range.[1,28–32] Consequently, moderate systemic folate depletion may be a cofactor in carcinogenesis, which has been described for cervical dysplasia.[4] The present study provides evidence of reduced folate levels in neoplastic colonic epithelial cells in the face of normal blood and adjacent tissue folate levels, but it does not indicate its relevance to malignant pathogenesis. Folate sequestration at the cell surface may confound these results. However, this is unlikely because the major cellular repositories for folate are cytosol, nucleus, and mitochondria.[33] Furthermore, gut epithelium is devoid of folate receptors.[34] Although such receptors have been noted in vitro, on some transformed colonic epithelial cells lines (Caco-2), such receptors are functional and rapidly internalize their ligand.

The lack of correlation between measures of systemic folate and colonic epithelial cell concentrations is similar to previous studies for both folate replete individuals and those using sulfasalazine.[12,35] However, perhaps more importantly, the similarity in results between control and adjacent tissues provides no evidence to suggest a field-folate deficiency in the colonic epithelium of those with or susceptible to the development of malignancy. They are in keeping with a previous study using maintenance sulfasalazine, which failed to find epithelial cell folate depletion in patients with ulcerative colitis, a condition associated with an increased risk for the development of CRC.[35] These results do not support the prophylactic use of folate supplements prescribed with the intention of correcting any presumed epithelial deficiency. However, the low serum folate levels noted in the tumor group may indicate a potential for folate supplements to overcome an undefined alteration in folate metabolism.

In conclusion, neoplastic colonic epithelial cells show a localized relative folate deficiency. However, adjacent normal-appearing epithelium shows no such depletion and has similar levels to those found in nontumor-bearing mucosa. This result suggests that any protective effect derived from dietary folate is mediated through mechanisms other than correction of cellular folate depletion.

## References


1. Giovannucci E, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, Rosner BA, Speizer FE, Willett WC. Folate, methionine and alcohol intake and risk of colorectal adenoma. J Natl Cancer Inst 1993;85:846–848.
2. Willett WC. Micronutrients and cancer risk. Am J Clin Nutr 1994; 59:1162s–1165s.
3. Adams RLP. Eukaryotic DNA methyltransferases—structure and function. Bioessays 1995;17:139–145.
4. Butterworth CE, Hatch KD, Macaluso M, Cole P, Sauberlich HE, Soong S, Borst M, Baker VV. Folate deficiency and cervical dysplasia. J Am Med Assoc 1992;267:528–533.
5. Kamei T, Kohno T, Ohwada H, Takeuchi Y, Hayashi Y, Fukuma S. Experimental study of the therapeutic effects of folate, vitamin A and vitamin $B_{12}$ on squamous metaplasia of the bronchial epithelium. Cancer 1993;71:2477–2483.
6. Cravo ML, Mason JB, Dayal Y, Hutchinson M, Smith D, Selhub J, Rosenberg IH. Folate deficiency enhances the development of colonic neoplasia in dimethylhydrazine treated rats. Cancer Res 1992;52:5002–5006.
7. Lashner BA, Heidenrich PA, Su GL, Kane SV, Hanauer SB. Effect of folate supplementation on the incidence of dysplasia and cancer in chronic ulcerative colitis: a case control study. Gastroenterology 1989;97:255–259.
8. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AMM, Bos JL. Genetic alterations during colorectal tumor development. N Engl J Med 1988;319:525–532.
9. Cravo M, Fidalgo P, Pereira AD, Gouveia-Oliveira A, Nobre Leitao C, Costa Mira F. Folate supplementation increases the degree of DNA methylation in patients with colonic neoplasms (abstr). Gastroenterology 1993;104:A394.
10. Holliday R. The inheritance of epigenetic defects. Science 1987; 231:163–170.
11. Kim Y, Giuliano A, Hatch KD, Schneider A, Nour MA, Dallal GE, Selhub J, Mason JB. Global DNA hypomethylation increases progressively in cervical dysplasia and carcinoma. Cancer 1994;74: 893–899.

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