

Folic acid deficiency and cancer: mechanisms of DNA instability

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Folic acid deficiency in humans has been linked with megaloblastic anaemia, neural tube defects in the neonate, and heart disease. Folate has also been implicated in the development of cancer, especially cancer of the colorectum. There appear to be two principal mechanisms through which low folate status may increase the risk of malignancy. Folate deficiency, by reducing intracellular S-adenosylmethionine (SAM), can alter cytosine methylation in DNA, leading to inappropriate activation of proto-oncogenes and induction of malignant transformation. Alternatively, folic acid is crucial for normal DNA synthesis and repair. Folate deficiency may cause an imbalance in DNA precursors, uracil misincorporation into DNA, and chromosome breakage. This chapter briefly describes the epidemiological data supporting the involvement of folic acid in the aetiology of cancer. It also assesses the evidence from cellular, animal and human studies that folic acid can modulate DNA by such mechanisms.

Folic acid, one of the B vitamins, acts as cofactor in numerous biochemical reactions through its ability to donate or accept one-carbon units. Mammals are unable to synthesise folic acid *de novo* and so must either obtain it from the diet or from microbial breakdown in the gut.

Folate deficiency has been reported to be the most common vitamin deficiency in the US, affecting 10% of the general adult population and up to 60% of juveniles or the elderly in low socio-economic groups^{1,2}. It has long been established that severe folate deficiency causes megaloblastic anaemia in humans, and that inadequate folate intake during pregnancy is related to neural tube defects in the newborn³. Poor folate status is also regarded as an independent risk factor for heart disease, mediated through elevated levels of the endothelial cell toxin, homocysteine⁴. More recently, however, folate deficiency has been implicated in the development of cancer, notably of the cervix, lung, breast, brain and colorectum.

The epidemiological evidence linking folate intake (in the form of fresh fruits and vegetables) with cervical dysplasia or cancer is at best circumstantial. It is further confounded by the presence in these foods of additional potential anticancer micronutrients such as vitamin C, vitamin E, and carotenoids⁵⁻⁷. Intervention studies have generated similarly

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confusing data. While several reports indicate that pharmacological doses of folate can improve or reverse cervical dysplasia in women using oral contraceptives, more recent large-scale placebo-controlled randomised trials have shown no protection⁸. Oral contraceptives have been postulated to induce a highly localised folate deficiency in the cervical epithelium, which may increase cancer risk. Similarly, cigarette smoke may cause localised folate deficiency in the bronchial epithelium. Serum folate levels are lower in smokers than in non-smokers and also in smokers with premalignant metaplasia^{9,10}. Supplementation with folate and vitamin B₁₂ reduces the severity of smoking-induced bronchial squamous metaplasia in humans and in chemical-induced metaplasia in rats^{11,12}. However, it may be unrealistic and undesirable to advocate folate supplementation as a potential prophylactic against lung cancer when we consider the extreme effect that cigarette smoking has on cancer risk.

The data linking folate intake with cancer of the colorectum are more convincing. Several questionnaire-based human trials have found an inverse association between reported folate intake and colorectal cancer incidence^{13,14}. Individuals with a high alcohol and low methyl (methionine and folate) intake are at a much greater risk of colorectal cancer than subjects with adequate intake¹⁵. Similarly, high alcohol, low folate and low protein intakes are inversely related to colon (but not rectal) cancer in men¹⁶. Surprisingly, in the same study, no relationship was found between serum folate as a biomarker and colon or rectal cancer. Intervention with folate has provided more conclusive findings. Folate supplementation protects against subsequent neoplasia in patients with ulcerative colitis¹⁷. Moreover, folate-mediated cytoprotection may be dose-dependent¹⁸.

Folate deficiency induces cytogenetic damage and mutations both *in vivo* and *in vitro*, with increases in DNA strand breakage, chromosomal aberrations and micronuclei formation. Mice made folate deficient over a 7 week experimental period show a 2-fold increase in micronuclei formation (Howell-Jolly bodies) in blood erythrocytes compared with folate-sufficient animals¹⁹. Chinese hamster ovary (CHO) cells cultured in folate-deficient medium have a 10-fold greater frequency of chromosomal aberrations (gaps, breaks and fragmented chromatids) than control cells²⁰. Moreover, folate deficiency can enhance the mutagenic potential of other genotoxins. CHO cells express an increased frequency of *HPRT*⁻ gene deletions (measured by PCR) when grown under folate-deficient conditions compared with cultures exposed to an alkylating agent alone²¹. Poor folate status has also been linked to cytogenetic damage in humans *in vivo*. Everson *et al*²² reported an exceptionally high frequency of micronucleated red blood cells in a patient with mild folate deficiency (1.9 ng/ml plasma folate). Supplementation with folic acid reduced the level of micronuclei to normal after only 8 days. Similarly, in a larger study of 150 individuals, subjects with low plasma and red blood cell folate or vitamin

B₁₂ had the highest observed micronuclei frequency²³. Common fragile sites (regions of the chromosome predisposed to breakage) and fragile sites associated with the human X chromosome-linked mental retardation syndrome, fragile X, are only expressed following culture in folate-deficient medium²⁴. There is some evidence that certain factors relevant to the expression of common fragile sites such as fra(3)(p14.2), are associated with activation of proto-oncogenes and progression and mortality of certain malignancies^{25,26}.

Essentially, there appear to be two mechanisms by which folic acid deficiency may increase the risk of cancer: namely by altering normal DNA methylation and by inducing an imbalance in DNA precursors leading to modified DNA synthesis and repair.

Folic acid status and DNA methylation

The function of folate in cellular methylation reactions has been covered elsewhere in this issue (DG Weir and J Little). However, the role of folate in DNA methylation will briefly be discussed.

The requirement for methyl groups to be used in cellular metabolism exceeds the normal dietary supply. Insufficiency is prevented by *de novo* methyl synthesis via one carbon-donation from the folate pool. Folic acid, in the form 5'-methyltetrahydrofolate (5'-methyl-THF), is essential in the conversion of methionine to its activated form, S-adenosylmethionine (SAM), via its ability to methylate homocysteine. SAM is the principal methyl donor in the majority of biochemical reactions, including the methylation of cytosine in DNA. Genes that are methylated at specific locations in the DNA molecule are either not transcribed or are transcribed at a reduced rate. In this way, site-specific DNA methylation controls gene expression. Alterations or disruption to DNA methylation may increase malignant transformation. Several studies have shown that specific human genes (*e.g.* growth hormone and proto-oncogenes) from tumour tissue (lung and colon) are substantially less methylated (hypomethylated) than genes from adjacent normal tissue^{27,28}.

If the level of 5'-methyl-THF, the main circulating form of folate, is lowered under conditions of folate deficiency, SAM quickly becomes depleted leading to a reduction in the methylation of cytosine in DNA¹⁴. This hypomethylation may result in upregulation of proto-oncogene expression and induction of cancer (Fig. 1). Indeed, methyl deficiency (lipotrope deficiency) has long been known to promote liver tumour development experimentally²⁹.

Wainfain and Poirier³⁰ have shown that rats fed a severely methyl-deficient diet (deficient in methionine, choline, folic acid and vitamin B₁₂) have a significantly reduced level of hepatic SAM and a reduced SAM to

SAH ratio. Global genomic DNA hypomethylation is induced within a very short time (1 week) on a methyl-deficient diet. The liver from lipotrope-deficient rats expresses elevated levels of mRNA for the proto-oncogenes *c-myc*, *c-fos* and *c-Ha-ras*; normal levels are restored when a control diet is fed. More importantly, methyl deficiency also increases DNA hypomethylation and DNA strand breakage in specific proto-oncogenes.

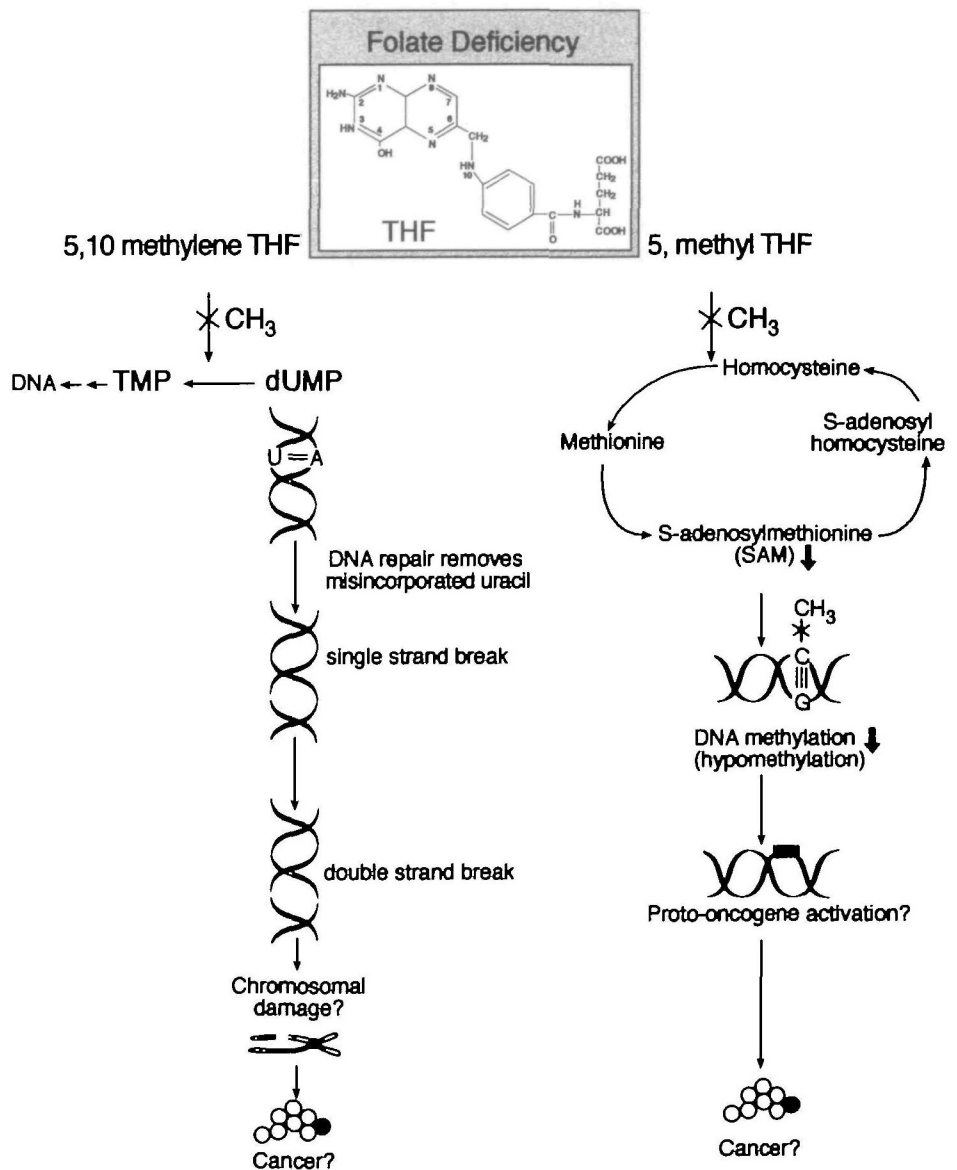


Fig. 1 Folate deficiency and DNA instability: potential mechanisms. Abbreviations: tetrahydrofolate (THF); 5,10 methylenetetrahydrofolate (5,10 methylene THF); 5' methyltetrahydrofolate (5' methyl THF); X denotes an inability to donate a methyl group (CH₃).

Pogribny and co-workers³¹ have shown, using a novel quantitative PCR method, that DNA strand breaks are induced in genomic DNA and, moreover, specifically within the p53 gene in liver from methyl-deficient rats. They hypothesise that DNA hypomethylation destabilises the DNA molecule making it more susceptible to attack. These studies have demonstrated that extreme methyl depletion damages the genome. Folate deficiency alone can also cause undermethylation of DNA. Rats fed a diet depleted of folic acid exhibit hypomethylated DNA after 4 weeks compared with pair-fed controls³². Folate deficiency appears specifically to hypomethylate p53 exons 6 and 7 from rat colon mucosa. Moreover, folate supplementation reverses chemically-induced hypomethylation in exon 8 of the p53 proto-oncogene³³. However, moderate folate depletion does not induce hypomethylation, either in total cellular DNA or in *c-myc* from liver or colon. Rats on a folate-deficient diet for 15 or 24 weeks had significantly lower plasma, colon and liver folate levels (90% depletion) than folate-sufficient rats. Moreover, levels of hepatic SAM were reduced and those of homocysteine, a functional indicator of folate status, were elevated. However, colonic SAM was unchanged and there was no difference in global or oncogene-specific DNA methylation³⁴. Why there is this inconsistency in the ability of folate deficiency alone to influence DNA methylation is unclear. It might reflect species or strain-specific differences in susceptibility to folate deficiency.

While it appears that low levels of folate are associated with an increased risk for colorectal cancer in man (see above), there is, as yet, no convincing evidence from human studies supporting the hypothesis that folic acid can influence DNA hypomethylation.

A population-based, incident case-control study of approximately 4000 cases and controls found no relationship between dietary intake of micronutrients and colon cancer risk. Likewise, there was only a trend of increasing risk between individuals with a high or low composite dietary profile based on alcohol, methionine, folic acid, vitamin B₁₂ and vitamin B₆ intake³⁵. Cravo and co-workers³⁶ found that colonic DNA was hypomethylated in patients with long-standing inflammatory bowel disease (IBD), a condition associated with an increased risk of colon cancer. Surprisingly, patients with IBD had higher blood and serum folate concentrations than controls. Furthermore, there was no significant correlation between endogenous DNA methylation levels (blood) and folic acid status and no effect of folate supplementation (5 mg daily for 6 months) on cytosine methylation. While this study suggests that folate status has no association with IBD and, moreover, does not influence DNA methylation, the authors reported that 75% of the patients had self-prescribed large doses of folic acid (10 mg daily) prior to the study. In addition, most of the patients (9/11) were concurrently treated with sulfasalazine, which significantly impairs folate uptake.

A significant criticism of studies which try to compare cancer cases and control subjects is that it is difficult, if not impossible, to discount any influence of the disease. For example, are folate levels generally low in individuals with cancer because a low dietary intake has increased the risk of cancer, or because having the disease results in low levels of folate in affected individuals, either through malabsorption or localised deficiency/disruption in metabolism? This has been addressed in part by Fang and co-workers³⁷ who measured folate status and DNA methylation in colorectal cancer patients and controls. Predictably, DNA isolated from cancerous tissue was hypomethylated compared with DNA from non-cancerous tissue. Plasma folate levels were lower in cases than in controls and, moreover, were lower in cancer patients who showed DNA hypomethylation than in cancer patients with normal methylation. However, the most convincing data come from a recent intervention trial on the effects of moderate folate depletion on DNA methylation in healthy postmenopausal women³⁸. Supplementing volunteers with folic acid at 56 µg/day for 5 weeks, then 111 µg/day for 4 weeks and finally 286–516 µg/day for 3 weeks varied folate intake. DNA methylation was measured in lymphocytes isolated from the women throughout the trial. A subclinical folate deficiency was created during the first 9 weeks of the study resulting in significantly decreased DNA methylation. This was reversed with 286–516 µg/day of folate. This is the first study to show that marginal folate deficiency can alter DNA structure in normal healthy individuals and that supplementation with folate can reverse DNA hypomethylation. In contrast, in another recent study of individuals with normal folate status, blood folate concentrations did not correlate with lymphocyte DNA methylation status, and folate supplementation did not alter methylation status³⁹.

The findings of a relationship between folate status and methylation patterns in individuals already diagnosed with cancer and, more importantly, in healthy normal individuals, provide credible evidence for a mechanism through which folate may modify DNA methylation and alter cancer risk. However, there is growing evidence that DNA hypermethylation can also occur during tumour development⁴⁰. While an increased risk for cancer can be explained by hypermethylation, and subsequently silencing, of tumour suppressor genes⁴¹, how folate deficiency might achieve this remains unclear.

Folic acid deficiency, DNA synthesis and repair

Folate is essential for the synthesis of purines and the pyrimidine nucleoside thymidine. Folate, in the form 5',10'-methylene tetrahydro-folate, acts as methyl donor for the enzyme thymidylate synthase which

converts deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP). It has been proposed that folate deficiency, by blocking the methylation of dUMP to TMP, can disrupt the balance of DNA precursors leading to accumulation of excess deoxyuridine triphosphate (dUTP) in the nucleotide pool. This may result in dUMP being misincorporated into DNA in place of thymidine, as DNA polymerases cannot distinguish between dUTP and dTTP. Uracil misincorporation into DNA is generally counteracted by deoxyuridine triphosphatase (dUTPase) and uracil DNA glycosylase (UDG). dUTPase specifically hydrolyses dUTP to pyrophosphate and dUMP thereby providing more substrate for thymidylate synthetase and minimising the risk of excess uracil accumulation. Uracil DNA glycosylase removes any misincorporated uracil from the DNA molecule⁴². Subsequent DNA repair enzymes act upon the DNA backbone to remove the base-free sugar, creating a small gap. The break is sealed by DNA ligase activity. However, if thymidine is continually limited under conditions of folate deficiency, uracil misincorporation and repair may occur repeatedly in what has been termed a 'futile' or 'catastrophic' repair cycle^{19,43-45}. Strand breaks, as intermediates in excision repair, may destabilise the DNA molecule, leading to chromosome aberrations and malignant transformation (Fig. 1). Uracil misincorporation does decrease DNA stability *per se*. Mutations in the *Escherichia coli dut* gene (coding for dUTPase) induce DNA fragmentation consistent with over-active excision repair stimulated by uracil misincorporation. Moreover, uracil DNA glycosylase-mediated repair of uracil lesions, spaced only 12 bp apart on opposite DNA strands of the plasmid pBR327, induces double strand breaks and gene deletions⁴⁶.

However, can poor folic acid status induce uracil misincorporation into DNA? Both *in vitro* and *ex vivo* studies of the influence of folate on megaloblastic anaemia and the expression of chromosome fragile sites suggest that it can. DNA extracted from cultured human myeloid cells (HL60) grown in folate-deficient medium contain higher levels of uracil and reduced concentrations of thymidine than DNA from cells grown in folate-sufficient medium⁴⁷. More importantly, bone marrow cells from patients with megaloblastic anaemia contain elevated levels of uracil, with approximately 30% of thymidines in the DNA being replaced by uracil⁴⁸. While folate deficiency in megaloblastic anaemia can induce uracil misincorporation into lymphocyte DNA, can this same mechanism account for the effect of folate deficiency on DNA stability and cancer risk? To answer this question, it is first necessary to develop a method to detect uracil in the DNA of single cells⁴⁹, since this makes it possible to assess the contribution of uracil misincorporation to the DNA instability observed in folate deficiency. The comet assay (single cell gel electrophoresis) in its original form measures DNA strand breakage in cells embedded in an agarose minigel. DNA breaks cause relaxation of the supercoiling in the

DNA molecule enabling free DNA loops to be pulled towards the anode during electrophoresis. Fluorescence staining enables DNA damage, seen as a 'comet tail', to be visualised and quantitated (Fig. 2). However, if the nucleoids are incubated before electrophoresis with bacterial UDG, which

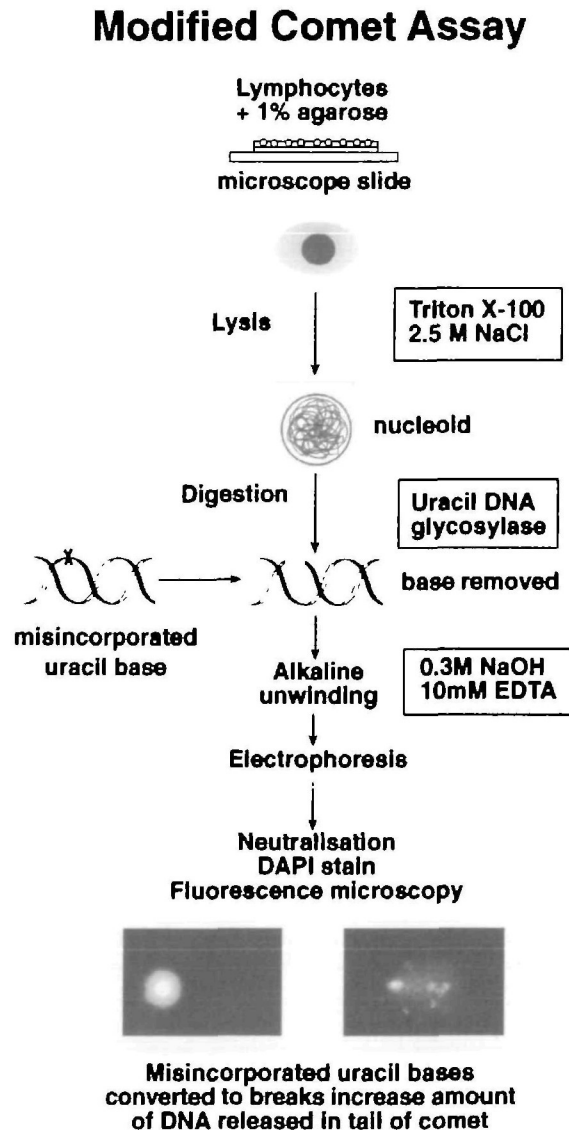


Fig. 2 Modified comet assay for detecting uracil in DNA. DNA damage is measured as strand breaks and misincorporated uracil. Cells are embedded in agarose on a frosted microscope slide. The slides are immersed in a high salt/detergent lysis solution to remove intracellular proteins. The slides are incubated with the bacterial DNA repair enzyme, uracil DNA glycosylase before unwinding in alkaline and electrophoresis. DNA damage is visualised by DAPI staining and fluorescence microscopy. The black and white images show comets from an undamaged and a heavily damaged lymphocyte.

Table 1 The effect of folate depletion on misincorporated uracil, oxidised pyrimidines and oxidised purines in human lymphocytes *in vitro*

Treatment	F ⁺	F ⁻
DNA strand breakage	19.8 ± 2.6	39.7 ± 4.8*
Uracil DNA glycosylase	31.9 ± 6.7	80.9 ± 11.7**
Oxidised pyrimidines	84.9 ± 15.4	78.9 ± 12.7
Oxidised purines	103.1 ± 29.9	125 ± 3

All units are arbitrary. Results are mean ± SEM ($n > 4$).

* $P < 0.007$, where P values refer to differences between DNA strand breakage in folate-sufficient (F⁺) and folate-deficient (F⁻) lymphocytes and ** $P < 0.005$, where P values refer to differences in uracil misincorporation in folate replete and deficient cells.

makes a break in the DNA where it recognises a uracil base, it is possible to detect misincorporated uracil specifically (Fig. 2). Using this technique we have found that normal human lymphocyte DNA contains small but detectable levels of uracil. Moreover, folate deficiency *in vitro* destabilises the DNA further. Lymphocytes cultured under conditions of folate deficiency show decreased proliferation following mitogenic stimulation. They also have an increased frequency of DNA strand breaks⁵⁰ and, most significantly, a 2–3-fold increase in the level of misincorporated uracil (Fig. 3A). Abnormal cell growth, DNA breakage and uracil misincorporation are inversely related to the concentration of folic acid available to the cells⁵⁰, suggesting that folate intakes which may prevent overt deficiency may not be sufficient for maintaining DNA stability. Uracil misincorporation in response to folate deficiency appears to be a relatively specific lesion, as poor folate status does not increase oxidative DNA damage. Lymphocytes grown in the presence or absence of folic acid contain similar levels of oxidised pyrimidines and purines (Table 1). Identical effects have been found in human colonocytes, which have similar levels of uracil and also show a 2–3-fold increase in uracil misincorporation following folate depletion (Fig. 3B). Human colonocytes are, of course, a better model system for investigating the mechanisms through which folate deficiency effects colorectal cancer. Folate deficiency *in vitro* also augments DNA strand breakage, apoptosis and death in CHO cells.^{51,52} More significantly, alterations in the deoxyribonucleotide pool, resulting in a 4–5-fold increase in the dUTP to dTTP ratio, increase the *hprt* mutation frequency in surviving cells⁵². These data linking uracil misincorporation with mutagenesis provide more convincing evidence for folate deficiency increasing malignant transformation.

As discussed previously, DNA repair provides one of the first lines of defence against DNA damage, mutagenesis and carcinogenesis. We have shown, using the comet assay, that folate deficiency decreases the ability

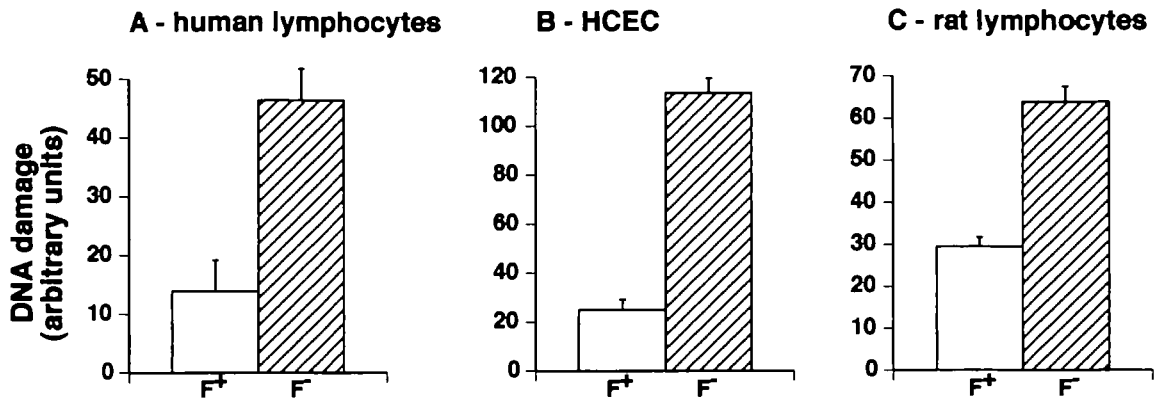


Fig. 3 Folate deficiency increases uracil misincorporation in human lymphocytes (A) and colonocytes (B) *in vitro* and in rat lymphocytes *in vivo* (C). Human lymphocytes ($n = 8$) or HCEC cells ($n = 4$) were grown in folate-sufficient medium (open bars) or folate-deficient medium (hatched bars) for 14 days. Rats ($n = 8$) were fed standard rat chow containing folic acid (open bars) or rat chow without folic acid (hatched bars) for 8 weeks. Uracil misincorporation is expressed as DNA damage in arbitrary units. Results are mean \pm SEM.

of human lymphocytes to repair oxidative damage⁵⁰. This is particularly important since diets that are lacking in folate will often be deficient in antioxidants such as vitamin C, vitamin E and carotenoids. Increased exposure to endogenous and exogenous reactive oxygen species, combined with specific DNA damage and inefficient repair as a result of folate deficiency, may augment DNA instability. Similarly, folate deficiency increases mutation frequency in response to gamma-irradiation and alkylation damage by inhibiting DNA repair⁵¹.

These *in vitro* studies indicate that folate deficiency can alter nucleotide precursor pools, induce uracil misincorporation and DNA strand breakage, and thereby reduce DNA stability. However, does this occur *in vivo*?

Early rat experiments found that folate deficiency increases DNA strand breakage in splenic lymphocytes and reduces thymidine synthesis. Intracellular NAD, the substrate for poly-(ADP ribose) polymerase is depleted, indicating up-regulation of DNA repair activity^{53,54}. Combined methyl deficiency (folate-, methionine- and choline-free diets) increases poly-(ADP ribose) polymerase activity and DNA strand breakage and induces apoptosis in liver sections. Hepatic dUTP levels were raised, TTP levels were reduced and the ratio of dUTP to TTP was increased 3-fold⁵⁵. Most importantly, methyl deficiency increased uracil levels in the liver 4-fold, as measured by the relative numbers of enzyme-induced DNA strand breaks after ExoIII/UDG treatment using the ROPS strand break assay⁵⁶. Similarly, uracil levels, detected using GCMS, is increased 2-fold in the liver of rats subjected to partial hepatectomy and treated with methotrexate. Removing 70% of the liver increases tremendously the demand

for nucleotides during liver regeneration, while methotrexate depletes folic acid, inhibiting the conversion of deoxyuridine to thymidine⁵⁷.

Total methyl depletion or methotrexate treatments combined with surgery are harsh regimens compared with folate deficiency alone. GCMS and the ROPS assay for detecting uracil in liver DNA are obviously more invasive and complicated techniques than the modified comet assay. It is, therefore, encouraging that we detect a similar increase in uracil misincorporation (2–3-fold) in lymphocytes from rats given a folate-deficient diet for 8 weeks (Fig. 3C). In agreement with our *in vitro* data, moderate folate deficiency (20% reduction in liver, plasma and erythrocyte levels) generates DNA strand breakage and uracil misincorporation, but does not affect oxidative DNA damage (Duthie *et al*, in preparation).

These animal studies confirm a central role for folate in maintaining the genome, but few comparable studies have been undertaken on humans.

Blount and co-workers⁵⁸ measured uracil levels and the frequency of micronucleated erythrocytes and reticulocytes in splenectomised individuals before and after folate supplementation. Individuals were classed as folate deficient if their red blood cell folate levels were below 140 mg/ml, or as normal if values were above this. Bone marrow and blood DNA from folate-deficient subjects contained 8–9 times more uracil than subjects with normal red blood cell folate. The frequency of micronucleated reticulocytes and erythrocytes was 3-fold greater in folate-deficient subjects than in individuals with acceptable folate levels. Supplementation with folic acid (5 mg/daily for 8 weeks) significantly increased plasma and erythrocyte folate levels in both folate-deficient and folate-sufficient volunteers. Moreover, uracil levels were decreased from 4.0×10^6 to 0.2×10^6 uracils per cell by folate supplementation in individuals with the lowest folate levels and from 0.5×10^6 to 0.14×10^6 uracils per cell in individuals with folate levels above 140 ng/ml. While supplementation with folate lowered the frequency of micronucleated cells by more than 50% in folate-deficient individuals, it did not change the frequency in subjects of normal folate status. This study provides good evidence that folate deficiency can induce uracil misincorporation and chromosomal damage in humans, and that intervention with folate can be beneficial (in terms of uracil misincorporation) both to individuals at risk and those with apparently acceptable folate levels. However, in a large cross sectional study of young adults with normal blood folate levels, lymphocyte micronuclei frequency was unrelated to folate status³⁹. Moreover, a 15% reduction in micronuclei frequency following supplementation with folate and vitamin B₁₂ (3.5 times the recommended dietary intake for 3 months) was associated with increased blood vitamin B₁₂ but not with changes in red blood cell folate. Uracil misincorporation was not determined.

Conclusions

Conventional epidemiological studies suggest that poor folate status is linked with an increased risk for specific epithelial cell cancers, most especially colorectal cancer. There are two principal mechanisms whereby folate is thought to modulate DNA stability and cancer incidence. The first hypothesis is that folate deficiency causes DNA hypomethylation and proto-oncogene activation. Data linking combined methyl deficiency with altered cytosine methylation and abnormal gene expression both *in vitro* and *in vivo* are convincing. Evidence proposing a similar effect for folic acid deficiency alone is less so. The second hypothesis is that folate deficiency induces continuous uracil misincorporation during DNA synthesis leading to a catastrophic DNA repair cycle, DNA strand breakage and chromosome damage. This is supported by evidence from cell and animal studies. Molecular epidemiological studies in humans indicate that folate deficiency leads to an increase in selective biomarkers for DNA damage. Moreover, folate supplementation can reduce DNA instability in folate-deficient subjects. While individuals with normal blood folate concentrations can benefit from increased supply of folic acid, it is debatable whether intervention with folate is necessary or advisable in the population as a whole. It may be judicious to promote increased folate intake for high-risk groups, but more rigorous human studies are required before scientifically based public health recommendations can be made about folate requirements of the general population.

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References

- 1 Senti RF, Pilch SM. Analysis of folate data from the 2nd national health and nutritional examination survey (NHANES-II). *J Nutr* 1985; 115: 1398–402
- 2 Bailey LB, Wagner PA, Christakis CJ *et al*. Folate and iron status and hematological findings in black and Spanish-American adolescents from urban low-income households. *Am J Clin Nutr* 1982; 35: 1023–32
- 3 Czeizel AE. Prevention of congenital abnormalities by periconceptional multivitamin supplementation. *BMJ* 1993; 306: 1645–9
- 4 Boushey CJ, Beresford SAA, Omenn GS *et al*. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA* 1995; 274: 1049–57

- 5 Verreault R, Chu J, Mandelson M *et al.* A case-control study of diet and invasive cervical cancer. *Int J Cancer* 1989; 43: 1050-4
- 6 Ziegler RG, Brinton LA, Hamman RF *et al.* Diet and the risk of invasive cervical cancer among white women in the United States. *Am J Epidemiol* 1990; 132: 432-44
- 7 VanEenwyk J, Davis FG, Colman N. Folate, vitamin C and cervical intraepithelial neoplasia. *Cancer Epidemiol Biomarkers Prev* 1992; 1: 119-24
- 8 Meyskens FL, Manetta A. Prevention of cervical intraepithelial neoplasia and cervical cancer. *Am J Clin Nutr* 1995; 62 (Suppl): 1417s-9s
- 9 Heimberger DC, Krumdieck CL, Dill SR *et al.* Localised folate deficiency and bronchial metaplasia in smokers: hypothesis and preliminary report. *Nutr Int* 1987; 3: 54-60
- 10 Piyathilake CJ, Hine RJ, Dasanayake AP *et al.* Effect of smoking on folate levels in buccal mucosal cells. *Int J Cancer* 1992; 52: 566-9
- 11 Heimberger DC, Alexander CB, Birch R *et al.* Improvement in bronchial squamous metaplasia in smokers treated with folate and vitamin B₁₂. *JAMA* 1988; 259: 1525-30
- 12 Kamei T, Kohno T, Ohwada H *et al.* Experimental study of the therapeutic effects of folate, vitamin A, and vitamin B₁₂ on squamous metaplasia of the bronchial epithelium. *Cancer* 1993; 71: 2477-83
- 13 Benito E, Stiggelbout A, Bosch FX *et al.* Nutritional factors in colorectal cancer risk: a case-control study in Majorca. *Int J Cancer* 1991; 49: 161-7
- 14 Giovannucci E, Stampfer MJ, Colditz GA *et al.* Folate, methionine and alcohol intake and risk of colorectal adenoma. *J Natl Cancer Inst* 1993; 85: 875-84
- 15 Giovannucci E, Rimm EB, Ascherio A *et al.* Alcohol, low-methionine-low-folate diets, and risk of colon cancer in men. *J Natl Cancer Inst* 1995; 87: 265-73
- 16 Glynn SA, Albanes D, Pietinen P *et al.* Colorectal cancer and folate status: a nested case-control study among male smokers. *Cancer Epidemiol Biomarkers Prev* 1996; 5: 487-94
- 17 Lashner BA, Heidenreich PA, Su GL *et al.* Effect of folate supplementation on the incidence of dysplasia and cancer in chronic ulcerative colitis. *Gastroenterology* 1989; 97: 255-9
- 18 Lashner BA, Provencher KS, Seidner DL *et al.* The effect of folic acid supplementation on the risk for cancer or dysplasia in ulcerative colitis. *Gastroenterology* 1997; 112: 29-32
- 19 MacGregor JT, Schlegel R, Wehr CM *et al.* Cytogenetic damage induced by folate deficiency in mice is enhanced by caffeine. *Proc Natl Acad Sci USA* 1990; 87: 9962-5
- 20 Libbus BL, Borman LS, Ventrone CH *et al.* Nutritional folate-deficiency in Chinese hamster ovary cells: chromosomal abnormalities associated with perturbations in nucleic acid precursors. *Cancer Genet Cytogenet* 1990; 46: 231-42
- 21 Branda RF, LaFayette AR, O'Neill JP *et al.* Effect of folate deficiency on mutations at the hprt locus in Chinese hamster ovary cells exposed to monofunctional alkylating agents. *Cancer Res* 1997; 57: 2586-8
- 22 Everson RB, Wehr CM, Erexson GL *et al.* Association of marginal folate depletion with increased human chromosome damage *in vivo*: demonstration by analysis of micronucleated erythrocytes. *J Natl Cancer Inst* 1988; 80: 525-9
- 23 MacGregor JT, Wehr CM, Hiatt RA *et al.* Spontaneous genetic damage in man: evaluation of interindividual variability, relationship among markers of damage and influence of nutritional status. *Mutat Res* 1997; 377: 125-35
- 24 Glover TW. FUDR induction of the X-chromosome fragile site: evidence for the mechanism of folic acid and thymidine inhibition. *Am J Hum Genet* 1981; 33: 234-42
- 25 LeBeau MM, Rawley JD. Chromosomal abnormalities in leukaemia and lymphoma: clinical and biological significance. *Adv Hum Genet.* 1986; 15: 1-54
- 26 Murata M, Otsuka M, Hayakawa Y *et al.* Expressivity of a common fragile site, fra(3)(p14.2) in patients with cancer and other diseases. *Jpn J Hum Genet* 1992; 37: 205-13
- 27 Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983; 301: 89-92
- 28 Fang J-Y, Zhu S-S, Xiao S-D *et al.* Studies on the hypomethylation of c-myc, c-Ha-ras oncogenes and histopathological changes in human gastric carcinoma. *J Gastroenterol Hepatol* 1996; 11: 1079-82
- 29 Ghoshal AK, Farber E. The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. *Cancer Res* 1984; 5: 1367-70

- 30 Wanfan E, Poirier LA. Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res* 1992; 52: 2071s-7s
- 31 Pogribny IP, Basnakian AG, Miller BJ *et al.* Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl-deficient rats. *Cancer Res* 1995; 55: 1894-901
- 32 Balaghi M, Wagner C. DNA methylation in folate deficiency: use of CpG methylase. *Biochem Biophys Res Commun* 1993; 193: 1184-90
- 33 Kim Y-I, Pogribny IP, Salomon RN *et al.* Exon-specific DNA hypomethylation of the p53 gene of rat colon induced by dimethylhydrazine: modulation by dietary folate. *Am J Pathol* 1996; 149: 1129-37
- 34 Kim Y-I, Christman JK, Fleet JC *et al.* Moderate folate deficiency does not cause global hypomethylation of hepatic and colonic DNA or *c-myc*-specific hypomethylation of colonic DNA in rats. *Am J Clin Nutr* 1995; 61: 1083-90
- 35 Slattery ML, Schaffer D, Edwards SL *et al.* Are dietary factors involved in DNA methylation associated with colon cancer? *Nutr. Cancer* 1997; 28: 52-62
- 36 Cravo M, Gloria L, De Sousa S *et al.* Folate status, DNA methylation and colon cancer risk in inflammatory bowel disease. *Clin Nutr* 1995; 14: 50-3
- 37 Fang J-Y, Xiao S-D, Zhu S-S *et al.* Relationship between plasma folic acid and status of DNA methylation in human gastric cancer. *J Gastroenterol* 1997; 32: 171-5
- 38 Jacob RA, Gretz DM, Taylor PC *et al.* Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J Nutr* 1998; 128: 1204-12
- 39 Fenech M, Aitken C, Rinaldi J. Folate, vitamin B₁₂, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis* 1998; 19: 1163-71
- 40 Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999; 21: 163-7
- 41 Esteller M, Sanchez-Cespedes M, Rosell R *et al.* Detection of aberrant promotor hypermethylation of tumour suppressor genes in serum from non-small-cell lung cancer patients. *Cancer Res* 1999; 59: 67-70
- 42 Lindahl T, Ljungquist S, Siegert W *et al.* DNA glycosidases; properties of uracil DNA glycosidase from *Escherichia coli*. *J Biol Chem* 1977; 252: 3286-94
- 43 Goulian M, Bleile B, Tseng B. Methotrexate-induced misincorporation of uracil in DNA. *Proc Natl Acad Sci USA* 1980; 77: 1956-60
- 44 Reidy JA. Folate- and deoxyuridine-sensitive chromatid breakage may result from DNA repair during G2. *Mutat Res* 1987; 192: 217-9
- 45 Reidy JA. Role of deoxyuridine incorporation and DNA repair in the expression of human chromosomal fragile sites. *Mutat Res* 1988; 211: 215-20
- 46 Dianov GL, Timchenko TV, Sinitsina OI *et al.* Repair of residues closely spaced on the opposite strands of plasmid DNA results in double strand break and deletion formation. *Mol Gen Genet* 1991; 225: 448-52
- 47 Wickramasinghe SN, Fida S. Misincorporation of uracil into the DNA of folate and B₁₂-deficient HL60 cells. *Eur J Haematol* 1993; 50: 127-32
- 48 Wickramasinghe SN, Fida S. Bone marrow cells from vitamin B₁₂- and folate-deficient patients misincorporate uracil into DNA. *Blood* 1994; 83: 1656-61
- 49 Duthie SJ, McMillan P. Uracil misincorporation in human DNA detected using single cell gel electrophoresis. *Carcinogenesis* 1997; 18: 1709-14
- 50 Duthie SJ, Hawdon A. DNA stability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes *in vitro*. *FASEB J* 1998; 12: 1491-7
- 51 Branda RF, Blickensderfer DB. Folate deficiency increases genetic damage by alkylating agents and gamma-irradiation in Chinese hamster ovary cells. *Cancer Res* 1993; 53: 5401-8
- 52 James SJ, Basnakian AG, Miller BJ. *In vitro* folate deficiency induces deoxynucleotide pool imbalances, apoptosis, and mutagenesis in Chinese hamster ovary cells. *Cancer Res* 1994; 54: 5075-80
- 53 James SJ, Yin L. Diet-induced DNA damage and altered nucleotide metabolism in lymphocytes from methyl-donor-deficient rats. *Carcinogenesis* 1989; 10: 1209-14
- 54 James SJ, Cross DR, Miller BJ. Alterations in nucleotide pools in rats fed diets deficient in choline, methionine and/or folic acid. *Carcinogenesis* 1992; 13: 2471-4

- 55 James JS, Miller BJ, Basnakian AG *et al.* Apoptosis and proliferation under conditions of deoxynucleotide pool imbalance in liver of folate/methyl deficient rats. *Carcinogenesis* 1997; 18: 287-93
- 56 Pogribny IP, Muskhelishvili L, Miller BJ *et al.* Presence and consequence of uracil in preneoplastic DNA from folate/methyl-deficient rats. *Carcinogenesis* 1997; 18: 2071-6
- 57 Blount BC, Ames BN. Analysis of uracil in DNA by gas chromatography-mass spectrometry. *Anal Biochem* 1994; 219: 195-200
- 58 Blount BC, Mack MM, Wehr CM *et al.* Folate deficiency causes uracil misincorporation into human DNA and chromosomal breakage; implications for cancer and neuronal damage. *Proc Natl Acad Sci USA* 1997; 94: 3290-5