Workshop Report

Folate and colo-rectal cancer risk

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The UK Food Standards Agency convened a group of expert scientists to review current research investigating folate and colo-rectal cancer risk. The workshop aimed to examine current research and establish research priorities. The timing of folate exposure with respect to carcinogenesis, as well as the dose and form of folate, were considered key issues for future research. Also, the need to study further the influence of genetically defined subgroups was highlighted for future research.

Colo-rectal cancer: Folate: Folic acid: Food Standards Agency

The UK Food Standards Agency (FSA) convened a workshop on 29 November 2006, on folate and colo-rectal cancer (CRC) risk. The results from recently completed studies, both FSA and non-FSA funded, were presented. The workshop was chaired by Professor John Baron.

Background

Folate is a generic term for a B-group vitamin (vitamin B_9) found widely in foodstuffs. Folic acid (pteroylmonoglutamic acid) is the synthetic form used in supplements and food fortification. Folates function coenzymatically in the transfer and processing of one-carbon units and play an important role in nucleotide synthesis, methylation, and the regulation of gene expression.

A meta-analysis of prospective cohort and case–control studies[1] concluded that folate has a modest inverse association with risk of CRC, although confounding by other dietary factors could not be ruled out. The inverse association observed was stronger for dietary folate than total folate (food and supplemental folate) intakes. Of the prospective cohort studies published since the meta-analysis, one has observed an inverse association between dietary folate intake and colon cancer risk[2], which was more pronounced among smokers, while, in contrast, a nested case–control study observed a bell-shaped association between plasma folate concentration and CRC risk; subjects with a plasma folate in the lowest concentration quintile had a lower CRC risk[3]. One other prospective study observed dietary folate not to be associated with the risk of CRC, but that intake of folate (and vitamin B_6) were inversely associated with risk among women not taking supplements[4].

In general, prospective cohort studies of an association between folate and colo-rectal adenoma risk provide some support for a role of folate in modulating colo-rectal carcinogenesis[5–7].

Methylenetetrahydrofolate reductase is a key enzyme regulating folate metabolism. Homozygotes (TT) for the *MTHFR* $677C > T$ gene polymorphism have been associated with a moderately reduced CRC risk[8], particularly among individuals who are folate-replete. Results from studies of the association between *MTHFR* $677C > T$ and risk of adenomatous polyps are inconsistent.

Overall the epidemiological evidence is suggestive of a modest inverse association between dietary folate and CRC risk, but confounding by other dietary factors may be a

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factor[9]. Polymorphisms in folate-metabolizing enzymes may also modify CRC risk in relation to folate intake.

Folate: a magic bullet or a double-edged sword for colo-rectal cancer prevention?

Professor Young-In Kim presented the evidence from animal studies investigating the role of folate in colo-rectal carcinogenesis. Studies using chemical colon carcinogens in rodent models have shown that a moderate degree of folate deficiency promotes, whereas modest levels of folic acid supplementation up to four times the basal daily requirement inhibits the development of CRC[10,11]. In contrast, folic acid supplementation exceeding the basal daily requirement by 20 to 10,000 times enhanced colo-rectal carcinogenesis in the chemical carcinogen rodent model of CRC[12–15].

In a genetically engineered murine model of CRC, the Apc^Min mouse, both semi-synthetic diets that contained one-third and twice the basal daily requirement for folic acid, respectively, increased the number of small intestinal polyps[16]. In the Apc^Min and Apc^Min × Msh2^{−/−} mouse models, moderate dietary folate deficiency enhanced, whereas modest levels of folic acid supplementation (four- to ten-times basal daily requirement) suppressed the development and progression of CRC, if folate intervention was started before the establishment of neoplastic foci in the intestine[17,18]. If, however, folate intervention was started after the establishment of neoplastic foci, the same degree of folate deficiency inhibited the progression and induced regression of the established neoplastic foci[17,18]. Furthermore, a potential tumour-promoting effect of folic acid supplementation on established neoplastic foci (aberrant crypt foci) was demonstrated in the azoxymethane-treated rat model[19].

Collectively, these animal studies suggest that folate possesses dual modulatory effects on colo-rectal carcinogenesis depending on the timing and dose of folate intervention. Folate deficiency has an inhibitory effect whereas folate supplementation may have a promoting effect on the progression of established colo-rectal neoplasms. In contrast, folate deficiency in normal colo-rectal mucosa appears to predispose it to neoplastic transformation, and modest levels of folic acid supplementation suppress, whereas supraphysiologic supplemental doses enhance the development of cancer in normal colo-rectal mucosa.

Folic acid in relation to DNA methylation and uracil misincorporation

Epigenetic events including genomic hypomethylation and aberrant hypermethylation of cytosine–guanine islands in the promoter regions of a sub-set of genes appear important in the development of human cancer[20]. Hypermethylation of cytosine–guanine islands results in the silencing of gene expression. Epigenetic alterations may play a central role in establishing the conditions under which tumourigenesis can become more likely, as epigenetic events in apparently normal tissue are likely to be present long before neoplasms are detectable[21]. Folate is required for the synthesis of S-adenosylmethionine, the primary methyl group donor for DNA methylation.

Thymidylate synthase converts deoxyuridine 5′-monophosphate (dUMP) to deoxythymidine-5′-monophosphate (dTMP) for the provision of thymidine. Folate is required as a cofactor for the reaction and deficiency can lead to uracil misincorporation associated with an increased risk of double-strand breaks leading to chromosomal breaks and genomic instability[22]. Thymidylate synthase is also a primary target for chemotherapeutic agents, e.g. 5-fluorouracil. Nucleotide synthesis and DNA methylation, therefore, provide relevant mechanisms linking folate metabolism to colo-rectal carcinogenesis.

Professor John Mathers presented the results from an FSA-funded trial of folic acid and riboflavin supplementation on colonic mucosa DNA methylation and uracil misincorporation. Three groups of volunteers were recruited to the study: individuals with no evidence of neoplasia (*n* 95); patients with colonic adenomatous polyps (*n* 102); and patients with CRC (*n* 74). At baseline, blood samples and rectal mucosal biopsies were collected, habitual diet was assessed and volunteers were genotyped for *MTHFR 677C > T* status. In a double-blind, placebo-controlled intervention trial, normal subjects and those with polyps received either placebo (*n* 48), 400 µg folic acid/d (*n* 49), 1200 µg folic acid/d (*n* 53), or 400 µg folic acid/d plus 5 mg riboflavin/d (*n* 54) for 50 d. A further set of blood samples and mucosal biopsies was collected at the end of the intervention period. At baseline, overall folate status was good and there was the expected inverse relationship between plasma folate and homocysteine. Mucosal folate concentration was positively correlated with plasma folate concentration. Methylation of the *P16* gene promoter region increased significantly with age and was higher in those with polyps and cancer than in normal subjects. Folic acid supplementation produced dose-dependent increases in plasma and mucosal folate concentrations and there were concomitant falls in plasma homocysteine concentration. Riboflavin supplementation increased riboflavin status. Methylation specific PCR (MSP) detected methylation in the promoter regions of the *P16*, *HPP1*, *MLH1* and *APC* genes in 83–100 % of volunteers. In contrast, only 3–12 % of volunteers had detectable methylation of the DNA repair gene *MGMT*. There were no significant effects of the interventions on DNA methylation (genomic or gene-specific) or on the misincorporation of uracil into DNA. The collection of colo-rectal mucosal cells for biomarker analysis using a 'brush biopsy', which was safer for, and more acceptable to, volunteers, was shown to be possible. There was no evidence of differences in response between normal subjects and those with polyps. This apparent lack of effect in both subject groups may be due to the relatively short intervention period or to the good folate status of the volunteers pre-supplementation.

Dr Ellen Kampman presented results from a case–control study and a human intervention trial examining the relationship between gene promoter methylation and intake of folate and folic acid respectively. Cytosine–guanine island methylation of *APC*, *p14*, *P16*, *MLH1*, *MGMT* and *RASSF1A* gene promoter regions was determined by MSP. In the case–control study analyses, patients with colorectal adenoma (*n* 149) and controls (*n* 286) with folate intake in the upper (>212 µg/d) or lower (<183 µg/d) tertile who were *CC* or *TT* homozygotes were included. MSP was performed for patients only, using paraffin-embedded colo-rectal adenoma tissue[23]. The percentages of promoter methylation ranged from 15·7 % to

64·2 %. Folate intake showed a non-significant inverse association with promoter methylation in colo-rectal adenomas in case–case comparisons, especially among those with the *TT* genotype. This appeared to be most pronounced for *MGMT*. The *MTHFR 677C > T* genotype alone was not associated with promoter methylation.

In the intervention trial, uracil misincorporation assays and MSP were performed on 'normal' rectal mucosa from eighty-six subjects with a history of colo-rectal adenomas who were randomly assigned to receive either a high dose of folic acid (5 mg/d) and vitamin B$_{12}$ (1·25 mg/d) or placebo for 6 months. Randomization was stratified for *MTHFR 677C > T* genotype. *MGMT* gene promoter methylation was significantly increased and uracil misincorporation and methylation of the other gene promoter regions examined showed a non-significant increase in the intervention group, which appeared to be more pronounced in subjects with the *TT* genotype.

These studies suggest that dietary folate may be inversely associated with *MGMT* promoter methylation in colo-rectal adenomas, especially for those with the *TT* genotype, but that a high dose of folic acid, in subjects with a recent history of adenomas, may adversely affect DNA methylation (silencing of the DNA repair gene, *MGMT*) and possibly DNA synthesis (increased uracil misincorporation), especially among *TT* homozygotes.

The application of microarray techniques to the measurement of gene-specific methylation status potentially offers the advantage of identifying in which genes methylation status is altered in disease processes, e.g. CRC, and in response to specific stimuli, e.g. diet. Professor Stephen Downes presented the preliminary results from an FSA-funded project to develop a methylation-sensitive microarray-based technique that could screen many genes simultaneously for changes in methylation. The technique employs standard gene expression cDNA microarrays: the number of genes screened is limited to the number of genes on the microarray. It involves cutting genomic DNA with methylation-sensitive and, separately, with methylation-insensitive, isoschizomers, adding linkers that are then annealed to the DNA, and PCR amplifying the products with primers at the linkers. DNA from the different isoschizomer digestions are labelled with different fluorochromes, and competitively hybridized to the array. The greater the number of methylated sites in or adjacent to any one gene, the weaker is the signal from the methylation-sensitive digest.

Folate depletion and repletion studies with SW620 colon tumour cells and the analysis of colon biopsy samples from patients with high and low folate status, and from subjects who participated in the trial presented by Professor John Mathers described earlier, led to the identification of several candidate genes, e.g. *H1F0*, whose methylation appeared to be sensitive to folate status. These findings are preliminary and validation with established techniques is ongoing.

Folic acid polyp recurrence trials

Professor Ken Muir presented results from the United Kingdom Colorectal Adenoma Prevention Consortium trial, in which subjects with a recent history of adenomas were randomly assigned to receive placebo (n 421) or 0·5 mg folic acid (n 432) daily with or without aspirin. The treatment

was for 3 years, after which subjects received a second follow-up colonoscopy. Folic acid supplementation had no effect on overall adenoma occurrence (relative risk (RR) 1·07, 95 % CI 0·85, 1·34) nor on risk of advanced adenoma occurrence (RR 0·98, 95 % CI 0·68, 1·40). The effect of folate metabolism genotypes on adenoma risk, in relation to folic acid supplementation, was examined in a subset of subjects (n 546). A significant reduction in recurrence risk was observed in subjects heterozygous for the methionine synthase reductase gene polymorphism (*MTRR 66A > G*) (RR 0·64, 95 % CI 0·46, 0·90) who received folic acid supplements, but not in those who did not receive folic acid; when homozygous subjects were included in the analysis, however, there was no significant effect[24]. No significant effect of folic acid supplementation on adenoma risk was observed in subjects analysed for other folate metabolism genotypes: *MTHFR 677C > T, MTHFR 1298A > C, MTR 2756A > G, TSER, TSER 3R G > C*, and *TS 1494del6*.

Professor John Baron presented results from the North American Aspirin–Folate Polyp Prevention Study, in which subjects with a recent history of adenomas were randomly assigned to receive placebo (n 505) or 1 mg folic acid (n 516) daily with or without aspirin. This trial was conducted in the context of the folic acid fortification of flour in North America, which is estimated to provide about 200 µg folic acid/d[25]. Initially designed with a 3-year treatment and follow-up, subjects were offered the option to continue randomized treatment for a second colonoscopic surveillance cycle (74 % accepted extended treatment). In the first surveillance cycle, adherence to study medications and avoidance of folic-acid-containing vitamin supplements was good, with compliance rates varying over time between 87 % and 96 % of subjects. In the first 3 years, folic acid supplementation had no effect on overall adenoma occurrence (RR 1·04, 95 % CI 0·90, 1·20) and showed a non-significant increase in the risk of advanced (RR 1·32, 95 % CI 0·90, 1·92) or multiple lesions (RR 1·20, 95 % CI 0·80, 1·81). In the second follow-up interval, the RR were higher. For all adenomas, the RR was 1·13 (95 % CI 0·93, 1·37), for advanced lesions it was 1·67 (95 % CI 1·00, 2·80) and for multiple adenomas it was 2·32 (95 % CI 1·23, 4·35).

Folic acid supplementation at 1 mg/d did not reduce the incidence of colorectal adenomas in subjects with a recent history of adenomas. There was some evidence that folic acid supplementation of 1 mg/d may increase the risk of multiple adenomas in these subjects, but this needs confirmation in other studies.

There is ambiguity about how to interpret the suggestions of increased risks during the second surveillance cycle. It could be that more prolonged folic acid supplementation is required for such an adverse effect, or that the original 3 years' supplementation was responsible for the increase, but it became evident only after a latent period of 3 years.

Discussion

The functions of folate in nucleotide synthesis and DNA methylation are considered central to a possible role in carcinogenesis and provide mechanisms by which it could be involved in both a beneficial and detrimental way[26]. Some animal studies

suggest that folic acid supplementation may help prevent the development of new cancers, but such studies also suggest that folic acid supplementation may promote the progression of existing premalignant/preneoplastic and neoplastic lesions. Furthermore, chemotherapeutic agents, e.g. methotrexate and 5-fluorouracil, target folate metabolism. A crucial issue with regard to this possible dual effect is the timing of exposure and dose of folic acid used.

The results from human intervention studies investigating an effect of folic acid supplementation on putative markers for CRC risk, e.g. DNA methylation and uracil misincorporation, are inconclusive. Studies investigating an effect of folic acid supplementation on global DNA methylation are complicated by differences in the analytical methods used; furthermore, most widely used methods for assessing genomic methylation provide no information on which cytosine residues within the genome are methylated and it is possible that reported changes in global DNA methylation are weighted towards changes within 'junk' DNA regions with no obvious implications for genomic stability, gene expression or cell function[27].

In the trial presented by Professor John Mathers there was no effect of folate supplementation on global DNA methylation in colo-rectal mucosa. In a previous trial in adenoma patients, high-dose folic acid supplementation (5 mg/d) for 6 months increased genomic DNA methylation in colonic mucosal biopsies, but when supplementation was continued for 12 months, genomic methylation was no different from that in placebo-treated subjects[28]. A similar trial in adenoma patients observed that, compared with placebo, folic acid supplementation (400 µg/d) increased genomic DNA methylation in leucocytes and non significantly in the colonic mucosa[29]. Other studies have shown that a few weeks of moderate folate depletion resulted in reduced global DNA methylation in circulating leucocytes, but any effect of folate repletion was inconsistent[30–32].

In healthy subjects, no effect on leucocyte genomic DNA methylation was observed after folic acid supplementation (1·2 mg/d for 10 weeks), but uracil misincorporation was reduced with the effect being more pronounced in those with lower erythrocyte folate concentration at baseline[33]. The study presented by Professor John Mathers, however, provides no evidence that folic acid supplementation (up to 1·2 mg/d for 50 d) alters colo-rectal mucosa uracil misincorporation. In contrast, results from the trial presented by Dr Ellen Kampman suggest that high-dose folic acid supplementation (5 mg/d for 6 months) may actually increase colo-rectal mucosa uracil misincorporation in subjects with a recent history of adenomas. This finding needs confirmation in a larger trial.

Two trials presented at the workshop assessed the effect of folic acid supplementation on gene-specific DNA methylation in the colo-rectal mucosa. While the trial presented by Professor John Mathers observed no effect of folic acid supplementation on DNA methylation in the genes examined (including *MGMT*), the trial presented by Dr Ellen Kampman showed high-dose folic acid supplementation to increase *MGMT* methylation status. This may be an adverse effect since *MGMT* methylation in the colo-rectal mucosa may result in gene silencing[34] and could represent a field effect predisposing to CRC[35].

The results from two polyp recurrence trials were presented at the workshop, neither of which demonstrated a beneficial effect on adenoma risk of folic acid supplementation (either 0·5 mg or 1 mg/d) over 3 years; however, in the North American trial the continued supplementation with 1 mg/d for a further 3–5 years was associated with an increased risk for more advanced lesions and multiple adenomas, suggesting folic acid supplementation may have promoted the progression of premalignant lesions. The United Kingdom Colorectal Adenoma Prevention Consortium trial provided no evidence of any adverse effects of 0·5 mg folic acid/d for 3 years on adenoma risk.

Overall, human intervention trials to date do not support a protective role of folic acid supplementation on CRC risk and there is some evidence to suggest an adverse effect of 1 mg/d or more when administered after colo-rectal neoplastic lesions have been established.

It has been suggested that if folate promotes the progression of preneoplastic lesions in human subjects, as demonstrated in some animal models, then a trial of recurrent polyps in subjects with a recent history of adenomas may be more relevant to investigating possible deleterious effects of folate supplementation[26]. Even conducting trials in adults without visible adenomas, however, raises the issue that many, perhaps most, would have transformed foci by the time they are middle-aged. This obviously raises ethical issues with regard to future trials and highlights the need to address the possibility that a key issue is the timing of exposure with respect to carcinogenesis.

To date all intervention trials investigating the relationship of folate to CRC risk have used the synthetic form of folate, folic acid. In human subjects, the degree to which folic acid is reduced and methylated in the mucosal epithelial cells to 5-methylenetetrahydrofolate remains unclear and dihydrofolate reductase activity appears to be more limited than in rodents[36]. A dose of about 260 µg has been reported to be the threshold at which folic acid starts to appear in the plasma postprandially[37]. There appears to be marked inter-individual variation in this response and repeated administration with smaller doses may result in the appearance of unmetabolized folic acid in plasma postprandially[38]. The concentrations of unmetabolized folic acid observed in the blood after the ingestion of supplements or fortified foods may have different effects on folate-binding proteins and transporters than naturally occurring folates[37]. Folic acid enters the one-carbon cycles at a different point to 5- methylenetetrahydrofolate and could, therefore, have different effects[26].

A cross-sectional study in the USA[39], where supplement use is common and the mandatory fortification of flour with folic acid was introduced in 1998, detected unmetabolized folic acid in fasting plasma samples of 78 % of subjects (n 105). The amount of unmetabolized folic acid in the plasma was inversely associated with natural killer cell cytotoxicity, an index of innate immune function and implicated in cancer risk[40].

Research recommendations

Future research should give consideration to the supplemental form, as well as the timing and dose, of folate employed. The inherent differences in folate absorption and metabolism

between rodents and human subjects[36] make it difficult to extrapolate the animal data to human subjects and further work is required to define dose–response effects. The further investigation of metabolic genotypes, e.g. *MTHFR 677C > T*, that may modulate the relationship between folate and CRC risk was also highlighted at the workshop. The prognostic significance of DNA methylation and uracil misincorporation for CRC risk, as well as their relationship to diet, still need to be determined.

Attendees

Professor John Baron, Dr Young-In Kim, Professor Joseph Rafter, Professor David Shuker, Dr Ellen Kampman, Professor John Mathers, Professor C Stephen Downes, Professor Ian Johnson, Dr Nigel Belshaw, Dr Elizabeth Williams, Professor Hilary Powers, Dr Susan Duthie, Professor John Toy, Professor Martin Wiseman, Dr Helga Refsum, Professor Richard Logan, Professor Ken Muir, Dr Paul Haggarty, Dr Andrew Povey, Professor Sheila Bingham, Professor Ian Rowland, Dr Alison Tedstone, Dr Elaine Stone, Ms Rachel Elsom, Dr Peter Sanderson.

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