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Folate, colorectal cancer and the involvement of DNA methylation

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Diet is a major factor in the aetiology of colorectal cancer (CRC). Epidemiological evidence suggests that folate confers a modest protection against CRC risk. However, the relationship is complex, and evidence from human intervention trials and animal studies suggests that a high-dose of folic acid supplementation may enhance the risk of colorectal carcinogenesis in certain circumstances. The molecular mechanisms underlying the apparent dual modulatory effect of folate on colorectal carcinogenesis are not fully understood. Folate is central to C_1 metabolism and is needed for both DNA synthesis and DNA methylation, providing plausible biological mechanisms through which folate could modulate cancer risk. Aberrant DNA methylation is an early event in colorectal carcinogenesis and is typically associated with the transcriptional silencing of tumour suppressor genes. Folate is required for the production of S-adenosyl methionine, which serves as a methyl donor for DNA methylation events; thereby folate availability is proposed to modulate DNA methylation status. The evidence for an effect of folate on DNA methylation in the human colon is limited, but a modulation of DNA methylation in response to folate has been demonstrated. More research is required to clarify the optimum intake of folate for CRC prevention and to elucidate the effect of folate availability on DNA methylation and the associated impact on CRC biology.

Colorectal cancer: Folate: Folic acid: DNA methylation

Colorectal cancer (CRC) occurs as a consequence of a complex series of genetic and epigenetic events leading to uncontrolled cell proliferation of malignant cells. Although the genetic events leading to neoplasia are well defined, an appreciation of the importance of epigenetic factors is relatively recent. Aberrant DNA methylation has been recognised as an early event in CRC and has been the focus of considerable research. As with other epigenetic events, DNA methylation is potentially reversible making aberrant DNA methylation in cancer a particularly attractive target for chemotherapeutics and chemoprevention. Diet is known to have a significant impact on risk of CRC and there is some suggestion of an inverse association between folate and CRC risk. Folate is a methyl donor for DNA methylation and consequently DNA methylation has received attention in attempts to elucidate the mechanism whereby folate may modify CRC risk. This review focuses on our current knowledge and understanding of the interaction between folate, CRC and the possible involvement of DNA methylation.

Colorectal cancer and its molecular classification

CRC is the third most common cancer in the world, accounting for 10% of all cancers and for approximately 20% of all deaths in the developed world[1]. It is an age-related disease with peak incidence in the seventh decade of life. The majority of CRC cases are sporadic; however, familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer are two forms of early onset hereditary CRC that account for approximately 1 and 5% of all CRC cases, respectively. FAP is characterised by the

Abbreviations: *APC*, adenomatous polyposis coli; CpG, cytosine–guanine; CRC, colorectal cancer; FAP, familial adenomatous polyposis.

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development of hundreds of adenomatous polyps (adenomas) in the colorectum at an early age, that progress to CRC if left untreated and are associated with a germ-line mutation in the adenomatous polyposis coli (*APC*) tumour suppressor gene. Hereditary non-polyposis colorectal cancer is characterised by DNA microsatellite instability as a result of defects in DNA mismatch repair genes. Understanding these gene defects in FAP and hereditary non-polyposis colorectal cancer has aided considerably in the understanding of CRC pathogenesis.

The genetic pathway of CRC was classically described by Fearon and Volgestein[2] who recognised that the disease was a multistep process that occurs as a consequence of an accumulation of genetic mutation in oncogenes and tumour suppressor genes (most notably *APC*, *KRAS* and *p53*). These mutations are believed to accumulate over several decades and are associated with the transformation of the normal colonic epithelium to an epithelium harbouring carcinoma. Adenomas are perhaps the most commonly accepted precursor lesion to CRC and are a key intermediate characteristic of this pathway, which is commonly referred to as the adenoma–carcinoma sequence[3]. The presence of an adenoma will increase the risk of CRC; however, only a small proportion of people with adenomas will go on to develop CRC demonstrating that not all adenomas transform into carcinomas[3].

Later research further classified CRC into two molecular subtypes: chromosomal instability and microsatellite instability. The chromosomal instability pathway is most closely related to the adenoma–carcinoma sequence; it is thought to account for the majority of CRC cases and is the recognised route of FAP cancers. The microsatellite instability pathway, classically observed in hereditary non-polyposis colorectal cancer involves defects in several genes involved in DNA mismatch repair, characterised by alterations in the length of repeat sequences of DNA known as microsatellites, and allowing genetic errors to persist and mutations to accumulate at an accelerated rate.

More recently, it has been recognised that epigenetic alterations are also associated with many cancers including CRC[4]. Epigenetics are heritable and potentially reversible changes in DNA expression that are not the result of a change in DNA sequence and include events such as histone modification, nucleosome positioning and DNA methylation[5]. Of these, it is DNA methylation that has been most strongly implicated in CRC biology. DNA methylation is the addition of a methyl group to a cytosine residue that occurs when the cytosine exists as a cytosine–guanine (CpG) dinucleotide. The bulk of such CpG dinucleotides can be found in the promoter region of genes where CpG dinucleotides cluster densely in what are known as CpG islands[6]. CpG dinucleotides also occur sporadically throughout the remainder of the DNA. Typically, CpG islands within the promoter region of an active gene are unmethylated, whereas the bulk of the DNA is methylated. Aberrant methylation (gene-specific promoter hypermethylation and global hypomethylation) has been observed early in the pathogenesis of CRC[7]. Although the impact of global hypomethylation is unclear, CpG promoter hypermethylation is most typically associated with a transcriptional silencing of the corresponding gene. A panel of genes, including the mismatch repair gene *MLH1*, have been identified as commonly being hypermethylated in CRC giving rise to a third molecular subtype of CRC known as the CpG island methylator phenotype[8].

Folate and colorectal cancer: the epidemiological evidence of an association

Folate is a water soluble vitamin found naturally in foods such as green leafy vegetables, citrus fruits, grains and offal. Folic acid is the synthetic form of the vitamin that is used in dietary supplements and as a food fortificant. A relationship between folate and CRC was first suggested by Lashner *et al.*[9] who reported a non-significant reduction in risk of CRC in patients with ulcerative colitis who received supplemental folic acid. A number of case–control and prospective cohort studies ensued. The evidence from case–control studies is inconsistent with some[10,11], but not all studies[12] indicating a protective effect of folate. Similarly, inverse associations between folate intake and CRC risk have been observed in many[13–17], but not all[18] prospective cohort studies. A meta-analysis of cohort and case–control studies reported a relative risk of CRC of 0·75 (95% CI 0·64, 0·89), in individuals consuming the highest compared with the lowest intake of dietary folate[19]. A subsequent analysis of prospective cohort studies led the WCRF/AICR (World Cancer Research Fund/American Institute for Cancer Research) expert committee to conclude that there was limited suggestive evidence that dietary folate protects against CRC[20]. This suggested modest benefit of dietary folate is supported by a more recent pooled analysis of thirteen prospective cohort studies that reported a relative risk of CRC of 0·92 (95% CI 0·84, 1·00) when comparing the highest *v.* the lowest quintile of dietary folate and 0·85 (95% CI 0·77, 0·95) for the total folate intake[21].

Folic acid supplementation has long been advocated for women for the prevention of neural tube defects[22]. The clear benefit of folic acid to the fetus has led many countries including the US to introduce a mandatory fortification programme resulting in an increase in folate status of the population[23] and the anticipated fall in neural tube defects[24]. However, some concerns of the impact of folic acid fortification were raised by an observational study published in 2007[25]. The authors identified a temporal association between folic acid fortification in the US and an increase in CRC rates. They estimated that there were between four and six additional cases of CRC per 100 000 of the population post-folic-acid fortification and suggested that the increase could result from an acceleration of progression of adenoma to carcinoma. A more recent prospective cohort study provides some reassurance that the level of folic acid fortification in the US is not detrimental[26]. The authors considered the relative risk of CRC pre- and post- the introduction of folate fortification and found similar inverse associations between the total folate intake and CRC risk both before and after the introduction of mandatory folic acid fortification. Further reassurance is provided by a recent re-examination of a large prospective cohort using data spanning 24 years, which found no

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evidence that high total folate intake increased the risk of CRC[27]. This study utilised dietary intakes that had been reported every 2–4 years and were able to examine the influence of the timing of folate exposure on CRC risk. They found inverse associations between CRC risk and the total folate intake 12–16 years prior to CRC diagnosis, but no association between CRC risk and more recent folate intake. In contrast, inverse associations existed between adenoma risk and both recent and long-term folate intakes. From this the authors suggested that dietary folate protects against the early, pre-adenoma stage of tumorigenesis[27].

What these observational studies have also revealed is that the association between folate and CRC is far from straightforward and the relationship between folate and CRC is modulated by many factors including alcohol and gender[15,28], and is further complicated by the impact of single nucleotide polymorphisms in genes involved in C_1 metabolism[29]. The C677T polymorphism in the 5, 10 methylenetetrahydrofolate reductase gene is perhaps the most widely documented polymorphism in this regard. There have been numerous studies reporting CRC associations with this single nucleotide polymorphism, which has culminated in a recent pooled analysis that has concluded that the C677T homozygous variant of this polymorphism confers protection against CRC[30]. Evidence is also emerging that the impact of folic acid on CRC risk depends on the genetic pathway involved. One cohort study has investigated the relationship between dietary folate and risk of CRC with and without the common *APC* mutation. Overall, they found no association between folate and cancer of the colon and rectum; however, when the cases were stratified according to the presence of an *APC* mutation, they found an inverse association with *APC* negative colon tumours in men, but a positive association with *APC* positive colon tumours[28], suggesting that high folate intake increases the risk of colon cancers involving mutated *APC*. Another observational study considered the relationship between folate concentration in the normal colorectal mucosa with risk and location of adenoma[31]. Folate concentration in the colorectal tissue is seldom measured, but is known to be responsive to folate supplementation[32] and is likely to be more biologically relevant than systemic folate status. They reported a significant inverse association between folate concentration in normal colorectal tissue and risk of proximal adenoma, but not with distal adenomas[31]. Since the majority of proximal adenomas are associated with the CpG island methylation phenotype this would suggest that folate protects against the specific CpG island methylation phenotype molecular subtype of CRC providing further evidence that the molecular classification of the tumour is important when designing and interpreting studies.

Folate and colorectal cancer: evidence from intervention trials

Given the long latency period of CRC in human subjects, it is difficult to conduct randomised controlled trials of folic acid with CRC incidence as the endpoint. However, as most CRC are thought to occur via the adenoma–carcinoma pathway[3], the recurrence of adenoma is generally accepted to be a good surrogate biomarker of CRC risk.

The evidence from human intervention trials is limited, and provides little evidence that folic acid supplementation protects against adenoma occurrence. In contrast, evidence has emerged from the Aspirin/Folate Polyp Prevention study that folic acid supplementation may be detrimental in certain individuals with a history of adenoma[33]. The folic acid arm of this double-blind placebo controlled trial involved 1021 people with adenoma. Participants were randomised to placebo or 1 mg folic acid. No differences were detected between treatment arms at the initial follow up after 3 years; however, at the second follow up after 3–5 years a significant increase in advanced adenoma was found in the folic acid treated group suggesting that high intakes of folate may accelerate the growth of adenomas. In agreement with the initial follow up results of that study the UKCAP (United Kingdom Colorectal Adenoma Prevention) study found no effect of a more modest 0·5 mg/d folic acid supplementation on adenoma recurrence after 3 years[34], whereas Wu *et al.*[35] reported no overall effect of 1 mg folic acid for a period between 5 and 6·5 years, but did report reduced adenoma recurrence in response to folic acid confined to a subgroup of subjects with low baseline folate status, particularly when coupled with a high-alcohol intake. Taken together these three studies suggest that in people with a history of adenoma folic acid supplementation is only beneficial in individuals with poor status and that supra-physiological doses may be harmful.

Further important evidence has been gathered using animal models of CRC. Earlier work in rodents demonstrated that folate deficiency was associated with increased development of colonic tumours in dimethylhydrazine-treated rats[36], and in the same model folic acid supplementation of up to four times dietary requirements was associated with a dose-dependent reduction in the number of macroscopic tumours[37]. In animals predisposed to spontaneously develop intestinal adenomas the timing of folate exposure has been shown to be critical. The $Apc^{Min/+}$ mouse carries an autosomal dominant nonsense mutation in the *APC* gene that predisposes the animals to adenomas in the intestinal tract and provides a model analogous to FAP. In this model supplemental post-weaning dietary folate was associated with a dose-dependent reduction in ileal polyps at 3 months, however, at 6 months there was no apparent benefit of supplemental folate and those animals fed with a folate deficient diet since weaning had the fewest ileal polyps[38]. Similar observations were made by Lawrance *et al.*[39] who reported that $Apc^{Min/+}$ mice receiving high-dose dietary folate since weaning had more small intestinal adenomas than animals fed with a folate deficient or control diet. This study also reported that prenatal folate exposure will modulate adenoma risk in $Apc^{Min/+}$ mice. No effect on adenoma number was observed when supplemental folic acid exposure was initiated prenatally and maintained postnatally, whereas folic acid deficiency initially induced prenatally reduced adenoma number[39]. Another study suggested that the apparent benefit of reduced folic acid post-weaning in $Apc^{Min/+}$ mice was confined to female mice[40]. Preneoplastic foci develop very early in the life

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of the $Apc^{Min/+}$ mice and the general interpretation of these data is that folic acid supplementation post-initiation of neoplasia will enhance, whereas folic acid deficiency will inhibit tumour progression.

Putative molecular mechanisms underlying the association between folate and colorectal cancer

Despite the wealth of epidemiological evidence that dietary folate modulates CRC risk the nuances of this association need further clarification, and our knowledge of the mechanisms involved also needs refinement. Folate has several important functions that are believed to account in part for the apparent association between folate and CRC. Folate is central to C_1 metabolism and is required for both the synthesis of DNA and for DNA methylation.

It is envisaged that the involvement of folate in DNA synthesis could account for how both folate deficiency and folate excess modulates CRC risk. 5,10 methylene tetrahydrofolate has an essential role in the formation of nucleotides and hence is required for DNA synthesis. 5,10 methylene tetrahydrofolate is the methyl donor needed for the conversion of deoxyuridine monophosphate to thymidine monophosphate required for the synthesis of thymidine. Folate deficiency results in a misincorporation of uracil instead of thymine. Attempts to repair the erroneous nucleotide are futile in a thymidine depleted environment, leading to DNA strand breaks and chromosome instability[41,42] associated with cancer. In contrast, excess folate may enhance tumorigenesis by supplying nucleotides and enhancing proliferation of pre-cancerous cells. The observation that folate can fuel the growth of neoplastic cells is well established and folate antagonists have been used for many decades as chemotherapeutic agents, most notably for the treatment of childhood leukaemia[43].

DNA methylation is the second putative mechanism through which folate is believed to modulate CRC risk. As previously described global hypomethylation and gene-specific promoter hypermethylation are features of CRC and a molecular subgroup of CRC displays the CpG island methylation phenotype. DNA methylation depends on the availability of folate; 5 methylene tetrahydrofolate is required for S-adenosyl methionine production, which is the universal methyl donor, providing methyl groups for methylation of DNA and other substrates. As folate is the methyl supplier for DNA methylation, this raises the possibility that exposure to folate will modulate the methylation status of DNA.

The role of folate in DNA synthesis is well established and we are fairly certain, at least in animal and *in vitro* models that folate deficiency will elevate uracil misincorporation and DNA strand breakage[44] and could account in part for the relationship between folate availability and CRC. Far less certain is whether folate availability has an appreciable impact on methylation status of DNA and how this contributes to CRC.

Modulation of DNA methylation by folate

Human studies investigating the impact of folic acid on colorectal DNA methylation are logistically challenging and consequently scarce. Such studies tend to be small and of limited duration. Nevertheless, inverse correlations have been observed between colonic mucosal genomic DNA methylation and folate status both in patients with colorectal neoplasia and in healthy subjects[45]. Another study reported increased leucocyte and colonocyte global genomic DNA methylation in response to a 10 week supplement of 400 μg folic acid/d in a small group of patients with colorectal adenoma[46]. In contrast, no such association between folate status and LINE-1 methylation (a marker of genome-wide methylation) was found in normal colorectal mucosa in healthy subjects[47]. However, low dietary folate has been shown to be associated with an increased risk of colon cancer with LINE-1 hypomethylation[48]. Wallace *et al.*[49] considered the association between demographic, diet and lifestyle characteristics with the methylation status of an age (*ERα*) and a cancer *(SFRP1)* related gene in rectal and colon biopsy samples from patients with adenoma randomised to the Aspirin/Folate Polyp Prevention study. Gene-specific promoter methylation was found to be associated with age, race and region of the bowel biopsied. They also reported a small, but significant increased promoter methylation of the two genes with increasing erythrocyte folate concentration, but no such relationship was evident with plasma folate status. Consistent with that observation, a randomised controlled trial of 5 mg folic acid and 1·25 mg vitamin B_{12} for 6 months in patients with a history of colorectal adenoma led to potentially deleterious increase in both promoter DNA methylation of genes implicated in CRC and in uracil misincorporation in the rectal mucosal[50].

Several studies have shown DNA methylation changes in animal models in response to altered dietary folate. Kim *et al.*[51] reported reduced methylation in the tumour suppressor gene, *p53*, but no change in genomic DNA methylation in the liver of rats fed a folate deficient diet for 6 weeks. However, global DNA hypomethylation has been reported in colon tissue of rats fed with a folate deficient diet for a far longer duration of 8 months[52]. Early life is key time for epigenetic changes and consequently it is important to understand the impact of folate availability *in utero* on long-term CRC risk. A series of manipulations of dietary folate have demonstrated that folate availability *in utero* and in early life can alter global DNA methylation in adult life[53,54]. In one such study reduced maternal dietary folate led to DNA hypomethylation in the intestinal tissue of the offspring, an effect not attenuated by folate repletion post-weaning[53]. In another study of azoxymethane induced tumorigenesis maternal folic acid supplementation at a level equivalent to fortification in the US was found to reduce tumour burden in the offspring, and was associated with significantly increased colorectal global DNA methylation[54].

So far, the investigation of folate and epigenetics has largely focused on gene-specific promoter methylation and global DNA hypomethylation. However, in recent years, further light has been shed on the complex epigenetic landscape. DNA methylation is now known to be associated with histone modification and nucleosome remodelling and the impact of methylation on the gene is

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no longer considered to be exclusively promoter methylation specific. Methylation patterns elsewhere in the DNA (CpG shores) are also linked with the CRC phenotype[55] and are thought to act as enhancers of transcription. See Baylin and Jones[56] for an excellent review of the topic. Fully understanding all aspects and interactions of the epigenome in the context of the folate and cancer story represents an important avenue of future research.

Conclusion

The relationship between folate and CRC is complex. Although the results from animal studies are not directly comparable with human subjects, they do imply that dose, duration and malignant status of the tissue will all determine the impact of folic acid availability on CRC risk. There is a suggestion from epidemiological studies that dietary folate confers modest protection against CRC; however, a discrepancy clearly exists between the apparent protective effect of folate in observational studies and the lack of benefit or possible detrimental effect of folic acid in intervention trials. This suggests that levels of folate that are achievable through the diet confer modest protection against CRC, whereas the high-doses of folic acid used in intervention trials have adverse consequences, particularly in people with existing adenoma. Such a scenario is indicative of a U shaped relationship between folate and CRC, and calls for an optimal dose of folate for cancer prevention to be defined. Finally, we are yet to fully elucidate the mechanisms involved in the apparent folate CRC relationship. DNA methylation is strongly implicated and is a biologically plausible mechanism; however, more evidence is needed before the contribution of folate to DNA methylation and associated CRC risk can be confirmed.

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