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Ageing, chronic alcohol consumption and folate are determinants of genomic DNA methylation, *p16* promoter methylation and the expression of *p16* in the mouse colon

Julia Sauer[1], Hyeran Jang[1], Ella M. Zimmerly[1], Kyong-chol Kim[1,2], Zhenhua Liu[1], Aurelie Chanson[1], Donald E. Smith[3], Joel B. Mason[1], Simonetta Friso[4] and Sang-Woon Choi[1]*

[1]*Vitamins and Carcinogenesis Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA, USA*
[2]*Department of Family Medicine, Mizmedi Hospital, Seoul, Korea*
[3]*Comparative Biology Unit, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA, USA*
[4]*University of Verona School of Medicine, Verona, Italy*

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Older age, dietary folate and chronic alcohol consumption are important risk factors for the development of colon cancer. The present study examined the effects of ageing, folate and alcohol on genomic and *p16*-specific DNA methylation, and *p16* expression in the murine colon. Old (aged 18 months; *n* 70) and young (aged 4 months; *n* 70) male C57BL/6 mice were pair-fed either a Lieber-DeCarli liquid diet with alcohol (18 % of energy), a Lieber-DeCarli diet with alcohol (18 %) and reduced folate (0·25 mg folate/l) or an isoenergetic control diet (0·5 mg folate/l) for 5 or 10 weeks. Genomic DNA methylation, *p16* promoter methylation and *p16* gene expression were analysed by liquid chromatography–MS, methylation-specific PCR and real-time RT-PCR, respectively. Genomic DNA methylation was lower in the colon of old mice compared with young mice ($P<0·02$) at 10 weeks. Alcohol consumption did not alter genomic DNA methylation in the old mouse colon, whereas it tended to decrease genomic DNA methylation in young mice ($P=0·08$). *p16* Promoter methylation and expression were higher in the old mouse colon compared with the corresponding young groups. There was a positive correlation between *p16* promoter methylation and *p16* expression in the old mouse colon ($P<0·02$). In young mice the combination of alcohol and reduced dietary folate led to significantly decreased *p16* expression compared with the control group ($P<0·02$). In conclusion, ageing and chronic alcohol consumption alter genomic DNA methylation, *p16* promoter methylation and *p16* gene expression in the mouse colon, and dietary folate availability can further modify the relationship with alcohol in the young mouse.

Alcohol: DNA methylation: *p16*: Folate: Ageing: Colon

Diet has a fundamental role as an environmental factor in determining the risk of colorectal carcinogenesis; for example, diets high in animal fat or red meat and low in fruits and vegetables tend to increase its risk[1–3]. Additionally, both epidemiological studies[4,5] and animal experiments[6] indicate that excessive alcohol intake plays an important contributory role in several common cancers, including those of the upper aerodigestive tract, liver, colorectum and breast[7,8].

In laboratory animals, chronic alcohol consumption has been observed to decrease genomic DNA methylation in colonic tissue, apparently by diminishing the availability of the proximate methyl donor, S-adenosylmethionine. It is thought that this effect is due to the increased utilisation of one-carbon donors and through its antagonistic effect on folate metabolism, especially the enzyme methionine synthase[9,10]. Folate is an essential cofactor in the synthesis of S-adenosylmethionine, the primary methyl donor for biological methylation. Since aberrations in DNA methylation (i.e. genomic DNA hypomethylation and gene-specific promoter hypermethylation) are an exceedingly common phenomenon in colorectal neoplasia[11,12], alcohol-mediated inhibition of methyl transfer reactions may be an important mechanistic avenue for alcohol-related cancers[8]. DNA methylation of the promoter region is an important mechanism for the control of gene expression: in many but not all instances hypermethylation represses and hypomethylation activates gene expression. Interestingly, neoplastic cells invariably demonstrate genomic hypomethylation of DNA and often demonstrate promoter hypermethylation of critical genes, such as tumour-suppressor genes[13,14]. Thus, activation of oncogenes by hypomethylation and the silencing of cancer-protective genes by hypermethylation may present a pivotal step in cellular malignant transformation.

Normally, tissue-specific methylation patterns established during embryonic development are highly preserved[15], but ageing and nutritional factors may modify these patterns[16,17].

Abbreviations: old, mice aged 18 months at the start of the study; young, mice aged 4 months at the start of the study.

* **Corresponding author:** Dr Sang-Woon Choi, fax +1 617 556 3234, email sang.choi@tufts.edu

It has been speculated that these age-related changes in methylation may contribute to the cellular hyperproliferation that occurs with ageing in some tissues, thereby promoting age-related diseases such as certain cancers[18,19]. Moreover, it has been shown that alcohol also modulates the methylation pattern of histones in conjunction with altered gene expression, and therefore altered methylation of molecules other than DNA may also be involved[20]. Like alcohol, ageing seems to promote genomic hypomethylation of DNA: rodent studies have observed this phenomenon in many organs, including the intestinal mucosa[21,22]. Moreover, ageing has also been observed to be associated with promoter hypermethylation of certain genes and repression of their expression in tissues such as the colon[23].

The p16 protein plays an important role in the regulation of the cell cycle, retarding proliferation. Loss of *p16* function by promoter methylation is a common epigenetic phenomenon in human colorectal carcinogenesis[24]. Moreover, the expression of the gene, which generally increases with ageing[21,25], is thought to play a mechanistic role in the ageing process because it promotes cell senescence and age-related decline in the functions of some organs[26].

The central role of 5-methyltetrahydrofolate in methyl transfer, and thereby in DNA methylation, might explain the relationship between folate status and the risk of colon cancer[14]. As explained above, ageing also induces genomic DNA hypomethylation and since rodent studies suggest that ageing directly impairs folate metabolism in the colon[27], it has been proposed that ageing might contribute to colonic carcinogenesis in part through its inhibition of folate metabolism[28]. Thus, we hypothesised that ageing, alcohol consumption and low dietary folate synergistically modify DNA methylation and the expression of the *p16* gene, both of which are highly associated with colonic carcinogenesis.

In the present study, we examined the impact of ageing and chronic alcohol consumption on select epigenetic features of DNA in the murine colon by measuring genomic DNA methylation, *p16* promoter methylation and the expression of *p16*. We further examined how alcohol consumption and low dietary folate, representing two nutritional factors associated with carcinogenesis that are biochemically interconnected, modulate the effects of ageing on DNA methylation in the colon.

Experimental methods

Animals and diets

The present study was reviewed and approved by the Animal Care and Use Committee of the USDA Human Nutrition Research Center on Aging at Tufts University. Seventy male C57BL/6 mice (old mice; aged 18 months; weight 30·6 (SE 3·6) g) and seventy male C57BL/6 mice (young mice; aged 4 months; weight 27·0 (SE 2·3) g) were used in the present study. Mice were obtained from Charles River Laboratories (Wilmington, MA, USA). The older 18-month-old mice were aged 20 months old by the end of the study. All diets were obtained from Dyets, Inc. (Bethlehem, PA, USA). No sulfonamide antibiotics were used to suppress colonic folate production.

The mice in each of the two age groups were randomly assigned to three groups of either twenty-three or twenty-four animals each. After 1 week of adaptation to the L-amino acid defined Lieber-DeCarli liquid control diet, one group was fed an L-amino acid defined Lieber-DeCarli alcohol diet[29]. The folate concentration in the Lieber-DeCarli was 0·5 mg folic acid/l liquid diet, which is equivalent to 2 mg folic acid/kg dry diet and accepted as the basal requirement for the mouse. Ethanol was present at a concentration of 6·2 % (v/v), comprising 36 % of total energy, and was gradually introduced over an adaptation period of 2 weeks. In week 1, the animals in the alcohol groups were given 12 % ethanol and in week 2 they were given 24 % to reach 36 % ethanol by the starting time point of the study. The second group received the same Lieber-DeCarli alcohol diet with folate depletion (0 mg folic acid/l liquid diet), which was introduced following the same schedule. The third group of mice received isoenergetic Lieber-DeCarli liquid control diets with 0·5 mg folic acid/l with an isoenergetic amount of maltodextrin. Water was not provided because the liquid diets provided enough fluid.

Individually housed mice were group pair-fed to decrease variability of energy and nutrient intake within and between dietary groups. The amount of food supplied to each diet group was matched to the mean daily food consumption of the group within the age category with the least food consumption. Therefore, all mice in each age group were offered the same volume of liquid daily and adjusted when necessary to ensure that all mice consumed equal volumes. All diets contained 16·11 % of energy as amino acids and 33·75 % of energy as fat. In the control diet, 47·7 % of the energy was derived from carbohydrates; in the alcohol diet this value was 11·7 %. We confirmed the concentration of 0·5 mg folate/l in the liquid diets with the microbiological assay. No folate was detectable in the folate-depleted diet.

Within the first week after completion of the adaptation period some of the old mice receiving 36 % ethanol started to die. Eight mice in the 36 % ethanol group and seven in the 36 % ethanol with folate depletion group died. No animals from the control group died. To prevent further loss during the study the ethanol concentration was reduced to 18 % by mixing equal portions of the ethanol and control diets, yielding an alcohol diet that was isoenergetic with the control diet. Due to inclusion of the control diet, the 18 % ethanol diet with folate depletion was enriched with folate (0·25 mg/l). Young mice were also fed the same alcohol diets. At 5 and 10 weeks, mice were killed and colon samples were harvested as previously described[27].

Hepatic folate concentration

Folate concentrations in liver and diets were measured by a conventional microbiological microtitre plate assay using *Lactobacillus casei* [30]. Due to the limited amount of colonic mucosal scrapings, we measured hepatic folate levels to determine systemic folate status.

Real-time RT-PCR assay for p16 *gene expression*

The expression of *p16* on transcriptional level was quantified using a Taqman Gene Expression Assay and an ABI Prism

7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Statistical analyses were performed using ΔCt (cycle threshold) after normalising gene expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ($\Delta Ct = Ct_{p16} - Ct_{GAPDH}$). Thus, lower ΔCt values indicate greater *p16* gene expression.

Genomic and p16 *promoter DNA methylation*

DNA methylation was measured using liquid chromatography–electrospray ionisation mass spectrometry as described[31]. DNA methylation of the *p16* promoter region was determined by bisulfite modification of genomic DNA and subsequent methylation-specific PCR, as described in detail elsewhere[21,32,33]. Briefly, 1 μg genomic DNA was bisulfite treated, desulfonated and recovered using a column-based kit (EZ DNA Methylation Gold; Zymo Research, Orange, CA, USA). Subsequently, DNA was amplified with a methyl-specific primer set (M forward, CGATTGGGCGGGTATTGAATTTTCGC; M reverse, CACGTCATACACACGACCCTAAACCG) and an unmethyl-specific primer set (U forward, GTGATTGGGTGGGTATTGAATTTTTGTG; U reverse, CACACATCATACACACAACCCTAAACCA) for the mouse *p16* promoter site[33]. Methyl amplification conditions included an initial denaturation at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 66°C for 1 min, and extension at 72°C for 1 min for forty cycles, followed by stabilisation for 8 min at 72°C. Unmethyl amplification followed the same procedure but with an annealing temperature of 64°C. The PCR products were separated by gel electrophoresis and stained with SYBR. The volume (intensity × mm^2) of methyl and unmethyl PCR products was quantified under UV light and promoter methylation status was relatively quantified as percentage of methyl band/(methyl band + unmethyl band)[34].

Statistics

To determine statistically significant differences between the age and diet groups at each time point, two-way ANOVA followed by Tukey's *post hoc* analysis was applied to determine the locations and level of significance. Logarithmic transformation was performed on skewed variables to normalise their distributions and statistical comparison was carried out on log-transformed data. The level of significance was $P<0{\cdot}05$ for all analyses. Values are presented as mean values with their standard errors.

Results

Animals

The body weights of old mice fed the control liquid diet remained stable throughout the experiment (Fig. 1). Initially, old mice fed the 18 % alcohol diets lost weight in week 1 but thereafter started to gain weight, and by 6 weeks there were no significant differences between the weights of the three groups of old mice. Despite the weight difference, all mice tolerated the liquid diets very well. During the 10-week experimental period two old mice in the control group died.

Young mice fed the control diet gained weight progressively during the experimental period. Like the older mice, young mice on the 18 % alcohol diets lost weight in week 1, but thereafter started to gain weight. This effect was observed in both the 18 % alcohol diet and the 18 % alcohol diet with reduced folate. By the end of the study, there remained a significant weight difference between the mice fed alcohol-containing diets and the control mice (Fig. 1). Among the young mice, one mouse died in the 18 % alcohol group and two mice died in the 18 % alcohol with reduced folate group before the end of the study. Necropsy of the mice that died early did not reveal any specific abnormality or disease.

Hepatic folate concentration

The mean hepatic folate concentrations (Table 1) were lower in old mice compared with young mice (10·4 (SEM 0·3) *v.* 11·5 (SEM 0·3) μg/g tissue; $P<0{\cdot}02$). Moreover, at 10 weeks the alcohol-fed old mice had a tendency of higher mean hepatic folate concentration compared with control old mice (11·4 (SEM 0·5) *v.* 9·3 (SEM 0·4) μg/g tissue; $P=0{\cdot}08$). Interestingly, the alcohol diet with reduced folate showed the same level of folate as the control. Hepatic folate concentrations did not differ among young mice groups.

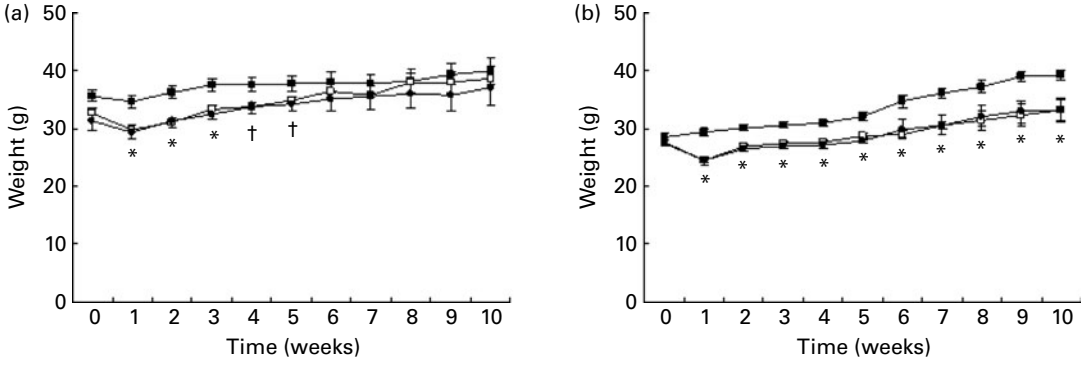

Fig. 1. Body weights in old (a) and young (b) male C57BL/6 mice fed a control diet (–■–), an 18 % ethanol diet (–□–) or an 18 % ethanol + folate-depleted diet (–●–). Values are means (*n* 15–24 for the first 5 weeks; *n* 8–13 for the second 5 weeks), with standard errors represented by vertical bars. * Mean value was significantly different from those of mice fed both ethanol-containing diets ($P<0{\cdot}05$). † Mean value was significantly different from that of mice fed the 18 % ethanol + folate-depleted diet ($P<0{\cdot}05$).

Table 1. Liver folate concentrations (μg/g tissue) in old (aged 18 months) and young (aged 4 months) male C57BL/6 mice fed a control diet, an 18 % ethanol-containing diet or an 18 % ethanol + low-folate diet for 5 and 10 weeks*

(Mean values with their standard errors)

	Old mice						Young mice					
	5 weeks			10 weeks			5 weeks			10 weeks		
Diet	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM
Control	11	10·3	0·8	11	9·3	0·4	10	11·9	1·2	13	11·1	0·4
18 % Ethanol	7	11·5	1·2	8	11·4†	0·5	10	12·3	0·7	12	11·0	0·5
18 % Ethanol + low folate	8	10·9	0·4	9	9·7	0·8	10	11·8	1·6	12	11·5	0·5

* The mean hepatic folate concentration of all old mice was significantly lower than that of all young mice (10·4 (SEM 0·3) *v.* 11·5 (SEM 0·3) μg/g tissue; $P<0·02$).
† Mean value was significantly different from that of the control old mice ($P=0·08$).

Genomic DNA methylation of the colon

Overall, genomic DNA methylation of the colonic mucosa was significantly lower in old mice compared with young mice by the end of the experiment (4·43 (SEM 0·04) *v.* 4·58 (SEM 0·04) %; $P<0·02$). This was also evident by the end of the experiment among the mice fed control diets: old mice fed the control diet showed a decreased colonic DNA methylation compared with that of young mice fed the same control diet ($P<0·02$). The presence of alcohol blunted the difference in methylation between the young and old: at 5 weeks the old mice fed alcohol with reduced dietary folate had a tendency of lower mean DNA methylation compared with the corresponding young mice ($P=0·08$). Also, at 10 weeks young mice fed alcohol demonstrated a tendency to have lower genomic DNA methylation compared with young control mice ($P=0·08$) (Table 2).

p16 *Promoter methylation and p16 expression*

Old mice had a dramatically greater degree of *p16* promoter methylation in all three diet groups at both 5 and 10 weeks ($P<0·001$) compared with the corresponding young groups, since young mice demonstrated low methylation status regardless of their diet. Neither alcohol consumption nor alcohol consumption with reduced dietary folate affected *p16* promoter methylation in either of the age groups (Fig. 2).

All old mice groups showed an increased *p16* expression compared with corresponding young mice groups ($P<0·05$) except alcohol-fed groups at 5 weeks (Table 3). Although there was no difference in *p16* gene expression among the three diet groups in old mice, young mice fed alcohol with reduced folate had decreased *p16* expression compared with the control group at both 5 ($P<0·02$) and 10 weeks ($P<0·01$). In contrast, young mice fed alcohol, but with sufficient folate, did not have significantly lower *p16* expression compared with control mice.

Interestingly, there is a positive correlation between *p16* expression and promoter methylation in the colon of old mice (Fig. 3; $P<0·02$) whereas in the young mice, among which colon *p16* promoter methylation status was invariably low, no significant relationship was observed.

Discussion

In the present study we used an older mouse model to examine whether genomic and *p16* gene-specific DNA methylation and *p16* gene expression, all of which are relevant to carcinogenesis, are modified by factors that are thought to have an impact on colorectal cancer risk: alcohol ingestion, dietary folate and ageing. Overall, we observed that older age has a remarkably robust and consistent impact on these molecular endpoints, whereas the effects of alcohol and mild folate depletion were more subtle and complex.

Ageing had a prominent role in determining genomic DNA methylation. Our present observations confirm a prior study of ours in which we demonstrated that genomic DNA methylation of the murine colon decreases with ageing[21], and this

Table 2. Genomic DNA methylation of colonic mucosa (% methylation) in old and young mice fed a control diet, an 18 % ethanol-containing diet and an 18 % ethanol + low-folate diet for 5 and 10 weeks*

(Mean values with their standard errors)

	Old mice						Young mice					
	5 weeks			10 weeks			5 weeks			10 weeks		
Diet	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM
Control	11	4·58	0·06	11	4·37†	0·08	10	4·37	0·15	13	4·75	0·08
18 % Ethanol	7	4·52	0·06	8	4·60	0·06	10	4·67	0·07	12	4·44§	0·10
18 % Ethanol + low folate	8	4·22‡	0·12	9	4·31	0·11	10	4·61	0·09	12	4·63	0·07

* DNA methylation in all old mice was significantly lower than that of all young mice (4·43 (SEM 0·04) *v.* 4·58 (SEM 0·04) %; $P<0·02$).
† Mean value was significantly different from that of the corresponding young mice ($P<0·02$).
‡ Mean value tended to be significantly different from that of the corresponding young mice ($P=0·08$).
§ Mean value tended to be significantly different from that of the young control mice ($P=0·08$).

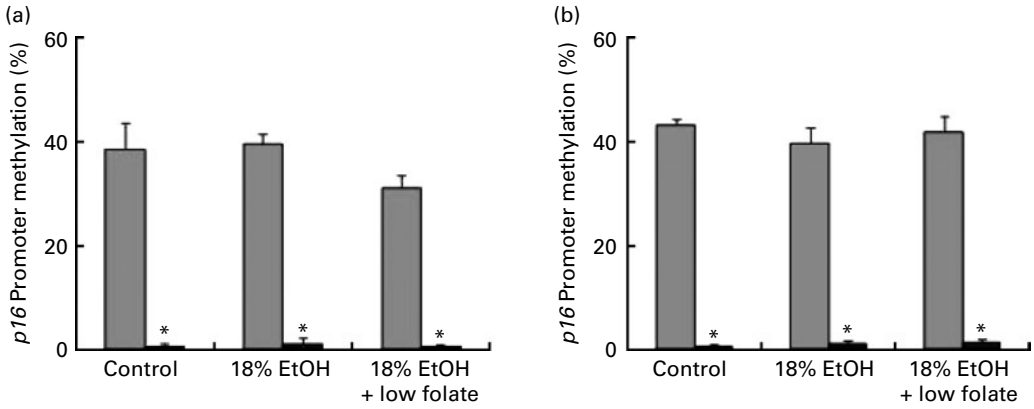

Fig. 2. Promoter methylation of *p16* in the colon of old (18 months; ■) and young (4 months; ■) mice fed a control, an 18 % ethanol-containing diet (18 % EtOH) or an 18 % ethanol + low-folate diet (18 % EtOH + low folate) for 5 (a) and 10 weeks (b). Values are means, with standard errors represented by vertical bars. * Mean value was significantly different from that of the old mice ($P<0{\cdot}001$).

age-related decline in genomic methylation of the colon appears to follow the same pattern in human subjects[35]. At 10 weeks, DNA methylation was significantly lower in all old mice compared with all young mice ($P<0{\cdot}02$).

Young mice fed alcohol demonstrated a tendency to have lower genomic DNA methylation compared with young control mice at 10 weeks. This observation is consistent with our previous study using young rats in which we found decreased genomic DNA methylation in the colon after 4 weeks of alcohol consumption[36]. Even though alcohol did not change genomic methylation in the old mice colon, it attenuated the ability to discern an effect of age on genomic methylation. The weak effect of alcohol on genomic DNA methylation, especially for the aged mice, may be related to alcohol exposure time that might be too short to affect genomic DNA methylation but more probably is related to the fact that alcohol was administered as 18 % of total energy, which is a much lower concentration of alcohol compared with 36 % of total energy that had been used in the former rat study[36].

Interestingly, the alcohol diet had a tendency to increase hepatic folate concentrations in old mice, which is consistent with what was observed in some prior rodent studies[36–38]. The observed increase in hepatic folate level might be related to the development of a methyl-folate trap, in which the methylated form of folate is accumulated in tissue and less utilised due to the inhibition of alcohol on methionine synthase[39]. Interestingly, at 5 weeks the old mice fed alcohol with reduced dietary folate had a tendency of lower mean DNA methylation compared with the corresponding young mice. It seems that they did not reach the significant level due to the mild degree of folate depletion in the diet. Since old mice also had lower overall hepatic folate concentrations compared with young mice ($P<0{\cdot}02$), diets may more influence folate level in old mice and reduced dietary folate decreased DNA methylation in old mice along with the methyl-folate trap induced by alcohol.

Gene-specific promoter hypermethylation appears to be an important biomarker of several human neoplasms, including colorectal cancer[40]. The *p16* gene product has a central function in controlling the cell cycle. Our observations indicate that promoter methylation of *p16* was not modified by chronic alcohol consumption for 10 weeks, regardless of if mild folate depletion was also present. On the other hand we found a prominent ageing effect on *p16* promoter methylation, which is consistent with a previous study of the older mouse colon[21], and which is a phenomenon that has been described in the older human colon as well[41]. Compared with ageing, a relatively short period of reduced methyl availability by alcohol consumption and reduced dietary folate might not be sufficient to perturb the system.

The hypothesis that hypermethylation of the *p16* promoter can diminish its expression in colonic carcinogenesis[42] does not appear in the older normal colon, because there is a significant increase in *p16* expression that parallels increases

Table 3. *p16* Expression (ΔCt) in colonic mucosa of old (18 months) and young (4 months) mice fed a control diet, an 18 % ethanol-containing diet and an 18 % ethanol + low-folate diet for 5 and 10 weeks

(Mean values with their standard errors)

	Old mice						Young mice					
	5 weeks			10 weeks			5 weeks			10 weeks		
Diet	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM
Control	11	17·2*	0·4	11	16·4*	0·3	10	19·2[a]	0·2	13	18·7[a]	0·3
18 % Ethanol	7	18·4	0·5	8	16·5*	0·5	10	19·3[a,b]	0·3	12	19·3[a]	0·3
18 % Ethanol + low folate	8	17·3*	0·3	9	17·0*	0·3	10	20·8[c]	0·4	12	20·5[b]	0·4

[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P<0{\cdot}05$).
* Mean value was significantly different from that of young mice in the corresponding dietary group ($P<0{\cdot}05$).

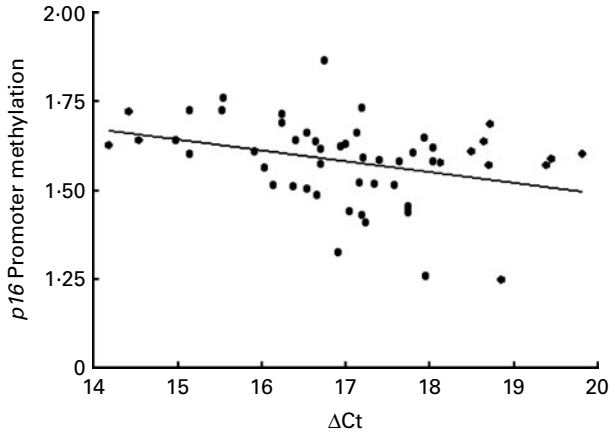

Fig. 3. Correlation ($P<0·02$) between *p16* expression status (ΔCt values) and promoter methylation (log-transformed) in the colon of old mice.

in promoter methylation in the old mice. Promoter methylation affects gene expression in conjunction with a number of other regulatory factors, such as histone modifications, methyl binding proteins and transcription factors, and therefore further studies will be required to explain the regulation of *p16* expression in the normal colon. In contrast, young mice had invariably low promoter methylation status and markedly lower *p16* expression, as was seen in our prior study[21]. In addition, the combination of alcohol and reduced dietary folate significantly diminished the gene's expression, an effect not seen with alcohol alone, demonstrating a synergy between alcohol and low dietary folate. This latter effect on *p16* expression is apparently conveyed by mechanisms other than promoter methylation.

Minor differences in weight gain due to alcohol administration feasibly introduced some confounding, although we believe that the effects were minimal. Both young and old mice that were fed alcohol gained less weight than mice fed the control diets during the 10 weeks of the study. This difference in the animal weights was expected[43] and has been previously ascribed to the different metabolic fates of nutrients that can be utilised as an energy source[44]. Old, but not young, mice that were fed alcohol caught up with the weight gain of their non-alcohol controls towards the latter half of the study, suggesting that old mice are better able to utilise energy from alcohol than the young. Perhaps the high metabolic demands for growth and development make the younger adult more vulnerable in this regard.

In conclusion, our observations indicate that older age is a major determinant of *p16* methylation and expression in the colon of the mouse and *p16* promoter methylation and expression are positively associated in the aged colon. Chronic alcohol consumption, which interferes with biological methylation, tended to reduce genomic DNA methylation in the colon of young mice, and the combination of alcohol and reduced dietary folate played minor roles in the colon of old mice. Young mice fed alcohol with reduced folate had decreased *p16* expression, indicating that dietary folate availability can further modify the relationship with alcohol in the young mouse. Our observations also underscore the idea that promoter methylation is not invariably the main factor determining *p16* expression by ageing and chronic alcohol consumption in non-neoplastic colon, even though promoter hypermethylation of *p16* is thought to play a role in the evolution of neoplasia in the colon by suppressing expression of the gene.

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All authors participated in the conception, design and performance of the study as well as interpretation of data and drafting the manuscript. J. S., H. J. and S.-W. C. also performed the generation, collection and analysis of data.

The authors do not have any competing interest.

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