Short Communication

Methylation status of CpG islands in the promoter region of genes differentially expressed in colonic mucosa from adenoma patients and controls in response to altered vegetable intake

Simone G. J. van Breda[1]*, Joost H. M. van Delft[1], Leopold G. J. B. Engels[2], Jos C. S. Kleinjans[1] and John C. Mathers[3]

[1]*Department of Health Risk Analysis and Toxicology, Maastricht University, Maastricht, The Netherlands*
[2]*Department of Gastroenterology, Maasland Hospital Sittard, Sittard, The Netherlands*
[3]*Human Nutrition Research Centre, School of Clinical Medical Sciences, Newcastle University, Newcastle upon Tyne NE2 4HH, UK*

Vegetables may protect against colorectal cancer (CRC) via changes in gene expression involved in anticarcinogenic mechanisms. There is considerable evidence that aberrant DNA methylation plays an important role in carcinogenesis. Furthermore, DNA methylation can be affected by dietary components. Therefore, in the present study, we investigated the DNA methylation status of CpG dinucleotides within the promoter region of the four genes protein kinase C β 1, ornithine decarboxylase 1, fos proto-oncogene and 5,10-methylenetetrahydrofolate reductase in the colon of female sporadic adenoma patients and healthy controls. These genes were chosen because their expression was modulated in response to altered vegetable intake, they are functionally relevant for CRC; they have CpG islands in their promoter region, and a methylation-specific restriction enzyme is available to permit quantitative assay. No significant differences in extent of methylation in colon DNA were detected for any of the four genes in both adenoma polyp patients and healthy controls after altering vegetable intake. Interestingly, before the intervention, ornithine decarboxylase 1 promoter methylation was lower in the colonic mucosa of the adenoma polyp patients when compared with healthy control subjects, which may explain the increased ornithine decarboxylase 1 activity in CRC reported in the literature. In conclusion, we found no evidence that changes in promoter methylation were responsible for differences in expression of four genes in the human colonic mucosa in response to altered vegetable intake. The mechanism(s) responsible for this altered gene expression and, indeed, potential effects on methylation of other genes remain to be determined.

Colorectal cancer: CpG island methylation: DNA methylation: Vegetable intake

A high intake of vegetables is associated with a reduced risk of numerous cancers, including colorectal cancer (CRC). The mechanisms by which vegetables exert their protective effects are diverse and generally involve changes in gene expression[1].

Control of gene expression is largely determined by chromatin structure. The accessibility of the chromatin is determined by the patterns of post-translational modifications of histones and by the methylation state of CpG dinucleotides in the promoter region of genes. Methylation of CpG dinucleotides is the main epigenetic modification of DNA[2].

There is considerable evidence that aberrant DNA methylation plays an integral role in oncogenesis[3]. Transcriptional silencing of genes involved in the regulation of cell proliferation, apoptosis and DNA repair by epigenetic mechanisms is critical in the pathogenesis of cancer, including CRC[4], and may be an early event predisposing to neoplasia[3].

The pattern of methylation is potentially reversible and offers an excellent target for modification of CRC risk by environmental factors, including diet[5]. However, information about the effects of dietary factors on gene-specific changes in methylation, especially in human subjects, is limited.

In a previous study, we examined the effect of altering vegetable intake on gene expression changes in the colon of female sporadic adenoma patients and healthy controls[6]. The present study was initiated to test the hypothesis that changes in expression of genes involved in CRC in response to altered vegetable intake are associated with changes in

methylation of the promoter regions of these genes, thereby contributing to understanding of the mechanism(s) of action responsible for the chemopreventive properties of vegetables. Therefore, we have investigated the methylation status of specific CpG within the CpG islands of the promoter region from a subset of these genes, by means of the modified combined bisulfite restriction analysis assay. Four genes were analysed: protein kinase C β 1 (*PKCB1*), ornithine decarboxylase 1 (*ODC1*), fos proto-oncogene (*C-FOS*) and 5,10-methylenetetrahydrofolate reductase (*MTHFR*). Within the group of differentially expressed genes, these four genes were selected for methylation analysis because only these genes met the following criteria: functionally relevant for CRC; gene expression significantly modulated; the presence of CpG islands in the promoter region of the gene and the availability of a methylation-specific restriction enzyme to permit quantitative assay.

Materials and methods

Subjects

The present study on DNA methylation is part of a project investigating the effects of increasing or decreasing the vegetable intake on gene expression in the colon mucosa of female adenoma patients and healthy controls. The characteristics of the study population and the study design have been published previously[6]. In short, the participants were recruited at the Maasland Hospital Sittard (The Netherlands). Eligible patients had a history of colorectal adenomas but no familial CRC syndrome or inflammatory bowel disease; eligible controls had no history of colorectal adenomas or other colorectal disorders. Both patients and controls were allocated at random into two groups of ten and four individuals respectively, receiving either a low (75 g/d) or high (300 g/d) vegetable diet (consisting of cauliflower, carrots, peas and onions) for a period of 2 weeks. Four rectal biopsies were collected at the first endoscopic examination and on the day following the last day of the intervention period. The present study was approved by the Medical Ethical Committee of the Maasland Hospital (Sittard, The Netherlands) and written informed consent was obtained from the participants before the start of the study.

DNA isolation

Four rectal biopsies (about 20 mg in total) from each subject were pooled and ground to a powder in a stainless steel mortar under liquid N_2 and homogenised in 800 μl TRIzol Reagent (GIBCO BRL, Life Technologies, Carlsbad, CA, USA). After isolation of RNA, which was used for gene expression analyses[6], DNA was extracted from the residuals according to the manufacturer's instructions. However, additional purification of the DNA appeared necessary. Therefore, DNA was treated overnight at 55°C with Proteinase K (Roche Diagnostics, Basel, Switzerland) (0·5 mg/ml in 2 mM-2-amino-2-hydroxymethyl-propane-1,3-diol-HCl, pH 7·4), followed by treatment with Rnase A (Roche Diagnostics) (100 μg/sample) and Rnase T1 (Roche Diagnostics) (50 units/sample) for 30 min at 37°C. Next, DNA was purified and concentrated using Dneasy® columns (Qiagen, Valencia, CA, USA). Quantity and quality of each DNA sample were assessed spectrophotometrically. The amount of DNA per sample varied from 25 to 68 μg.

Analysis of CpG island methylation: modified combined bisulfite restriction analysis assay

A modified combined bisulfite restriction analysis assay[7] was used to determine the methylation status of CpG within the CpG islands in the promoter region of the four genes *C-FOS* (at +40 from the start of transcription), *PKCB1* (at +166), *ODC1* (at +82) and *MTHFR* (at +384).

Bisulfite modification. Genomic DNA (2 μg) was treated with sodium bisulphite according to the method of Raizis *et al.* [8].

Polymerase chain reaction amplification. By means of PCR, components of the CpG islands within the promoter regions of the four genes were amplified. An approximately 600 bp long DNA sequence upstream of the transcriptional start area of each gene was selected as the promoter region using Ensemble (http://www.Ensemble.org/). The presence of CpG islands within these promoter sequences was predicted using the online software program Methprimer (http://www.urogene.org/methprimer/). PCR reactions (25 μl), containing 12·5 μl 2 × HotStarTaq Master Mix (Qiagen), 4 pmol of each forward and reverse primer (MWG Biotech, Milton Keynes, Bucks, UK), 1 μl bisulfite modified DNA and supplemented with $MgCl_2$ to a final concentration of 2 mM, were subjected to the following cycling conditions: one cycle at 95°C for 15 min, thirty-five cycles at 95°C for 30 s, annealing temperature for 1 min (50°C for *PKCB1*, *C-FOS* and *MTHFR*; 57°C for *ODC1*), 72°C for 1 min, followed by a 5 min extension at 72°C. PCR products were stored at 4°C until restriction analyses.

Restriction analyses. PCR mixtures were digested overnight with 5 units of the restriction enzyme (*Nru* I for *PKCB1*; *Taq* I for *ODC1*, and *Acl* I for *C-FOS* and *MTHFR*) (New England Biolabs, Ipswich, MA, USA), according to the conditions specified by the manufacturer. Restriction enzymes at a methylation-sensitive CpG site were selected using the online program webcutter (http://rna.lundberg.gu.se/cutter2/). Digested PCR products were separated on gel and stained with SYBR green I (Molecular Probes, Invitrogen, Eugene, OR, USA). The DNA SYBR green fluorescence was captured using a CCD camera fitted with UVIphoto software and band intensities were quantified using UVIband (Uvitec Limited, Cambridge, Cambs, UK). The proportions of methylated and unmethylated DNA were calculated from the relative intensities of cut and uncut PCR product (total intensity set at 100 %). Next, a methylation difference for each subject and gene was calculated by subtracting the proportion of methylated alleles before the intervention from the proportion of methylated DNA after the intervention.

Statistical analysis

Statistical analysis of the data was carried out using SPSS (version 12.0 for Windows; SPSS Inc., Chicago, IL, USA). Potential differences in methylation status were assessed using ANOVA for patients *v.* controls, for increased (high)

v. decreased (low) vegetable intake, and for patients and controls within each vegetable group. All tests were two-sided and adjusted for differences in age. A *P* value <0·05 was considered to indicate statistical significance.

Results

Methprimer predicted CpG islands within the promoter region of all four genes. PCR amplification of a region of these CpG islands was successful (data not shown). Next, PCR products were digested using a restriction enzyme that cuts at a predicted methylation site, and separated on a gel (data not shown). The extent of methylation at the chosen CpG site in the promoter region of the four genes for both patients and controls before and after the dietary intervention is shown in Table 1. This Table also shows the fold changes of the expression of these four genes after the vegetable intervention and the difference in promoter methylation (after − before) which facilitates direct comparison between changes in DNA methylation and in gene expression by an altering intake of vegetables.

For both *PKCB1* and *CFOS*, the extent of methylation in all samples was too low to quantify. Although the extent of methylation was quantifiable for *ODC1* and *MTHFR*, in more than 90 % of the samples the level of methylation was less than 7 %. No significant differences in the extent of methylation in colon DNA were detected for any of the four genes in either patients or controls after the vegetable diet intervention (data were adjusted for age). However, at baseline, the extent of methylation in the promoter region of the *ODC1* gene was significantly higher in healthy controls (5·3 (SEM 1·1) %) compared with that in adenoma patients (2·0 (SEM 0·5) %) (*P*<0·01).

Discussion

Little is known about the mechanisms responsible for the regulation of expression of any of the four genes *PKCB1*, *C-FOS*, *ODC1* and *MTHFR*, or how the expression can be affected by exogenous factors such as diet. However, the coupling of promoter methylation to gene expression silencing is well established[9] and other regulatory mechanisms have been reviewed by Mathers[10]. Furthermore, although biopsies were taken from phenotypically normal tissue, gene expression differences could be detected, indicating that changes in CpG island methylation may be a possible mechanistic explanation.

In the present study, methylation in the promoter region of *PKCB1* and *C-FOS* was below the limit of detection. Consequently the change in expression of these genes by the altering vegetable intake cannot be explained by a change in DNA methylation. No other studies have investigated the effect of diet on either methylation or mRNA expression of *PKCB1*. Two previous studies have investigated DNA methylation changes in relation to *C-FOS* gene expression change[11,12]. However, this research was carried in rat liver and no comparable human data are available.

ODC1 catalyses the first step in the polyamine biosynthetic pathway, a highly regulated pathway associated with rapid growth states, including higher CRC risk[13]. Although low levels of *ODC1* promoter methylation could be quantified in the colonic mucosa of both healthy control subjects and adenoma polyp patients, there was no evidence that down-regulation of expression by vegetable intake, as reported in our previous study[6], coincided with a significant change in methylation. Several studies have investigated the methylation status of the promoter region of the *ODC1* gene[14–16] and found that the promoter region was extensively methylated[14–16]. Furthermore, hypomethylation within and/or around the *ODC1* gene was associated with an enhanced accumulation of *ODC1* mRNA[14,15]. It must, however, be noted that most of these studies investigated the methylation status of the *ODC1* gene in tumour cells, and so do not provide information relevant to healthy normal tissue. To our knowledge, only one previous study has examined *ODC1* methylation status in humans[14]. *ODC1* was less methylated in leukaemia cells than in leucocytes from healthy subjects. We now report that the methylation of the *ODC1* gene at target tissue level, i.e. in the colonic mucosa of healthy subjects, was higher than that in the adenoma patients who are at higher risk of CRC. This difference would be expected to contribute to reduced *ODC1* expression, ODC1 activity and polyamine concentrations in healthy mucosa.

Although methylation of the promoter region of the *MTHFR* gene was quantifiable in mucosa from both healthy controls and adenoma polyp patients, the levels of methylation were low (and similar) in both subject groups. The up-regulation of *MTHFR* gene expression in the colon of healthy controls cannot be explained by a change in methylation status of the promoter region of this gene. We are not aware of other published studies that have investigated the methylation status of the promoter region of MTHFR in response to dietary intervention.

There are several possible explanations for the absence of methylation changes in the promoter region of the investigated genes in response to the dietary intervention. First, most of the previous dietary studies that have found an effect on DNA methylation have modified methyl group supply specifically. Most of these studies have been undertaken in rodents in which dietary methyl (folate, choline and methionine) deficiency causes global or specific DNA hypomethylation[17–19]. The results of more recent studies of manipulation of folate supply in humans and implications for CRC have been reviewed by Mathers[20]. It has been suggested that dietary interventions which do not involve major changes in methyl group supply are unlikely to have a major effect on DNA methylation[21], but this has yet to be evaluated. Second, the time-scale for the establishment of DNA methylation changes might be longer. The third possibility is that the gene expression changes that we have observed are probably not the result of changes in DNA methylation. Other epigenetic markers such as changes in histone acetylation patterns could have contributed to the observed effect on gene expression, as could changes in the supply of transcription factors due to the dietary intervention[10].

This is the first study in which the methylation status of genes in response to dietary intervention has been investigated in the colonic mucosa of human subjects. We have shown the presence of low levels of methylation of the promoter region of the *ODC1* and *MTHFR* gene, in both healthy control subjects and adenoma polyp patients. Interestingly, the extent of *ODC1* promoter methylation was lower in the colonic

Table 1. Effect on gene expression and on DNA methylation extent (%) at a single CpG site in the promoter region of four genes significantly modulated by vegetables

(Mean values with their standard errors)

	Effect on gene expression†								DNA methylation (%)*																				
	Healthy controls (n 8)				Patients (n 20)				Healthy controls										Patients										
	LV		HV		LV		HV		Baseline methylation‡ (n 8)		LV (n 4)				HV (n 4)				Baseline methylation‡ (n 20)		LV (n 10)				HV (n 10)			
											After§		Difference‖		After§		Difference‖				After§		Difference‖		After§		Difference‖	
Abbreviation by NCBI¶	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
PKCB1	0·8 (up-regulation)	0·2			0·4 (up-regulation)	0·2			ND		ND		ND		ND		ND		ND		ND		ND		ND		ND	
ODC1			−1·4 (down-regulation)	0·4			−0·8 (down-regulation)	0·2	5·3	1·1	5·1	2·0	−0·3	1·3	5·6	1·3	−2·4	1·7	2·0**	0·5	3·1	0·7	−1·1	0·9	0·9	0·5	2·5	0·9
C-FOS			−2·1 (down-regulation)	0·5			−0·9 (down-regulation)	0·2	ND		ND		ND		ND		ND		ND		ND		ND		ND		ND	
MTHFR	0·6 (up-regulation)	0·1							2·8	0·9	1·1	0·8	0·3	0·9	4·6	1·2	−0·6	1·5	3·4	0·6	3·1	0·9	0·6	1·0	3·6	0·7	−0·2	1·0

LV, low vegetable group (75 g/d); HV, high vegetable group (300 g/d); NCBI, National Center for Biotechnology Information; PKCB1, protein kinase C β 1; ND, not detectable; ODC1, ornithine decarboxylase 1; C-FOS, fos proto-oncogene; MTHFR, 5,10-methylenetetrahydrofolate reductase.

* Extent (%) of methylation at a single CpG site in the promoter region of four genes whose expression was significantly modulated by vegetable intake.

† Gene expression changes (mean log(2)-fold changes) in colon mucosa after an intervention with vegetables during 2 weeks[6]. Empty cells indicate that there was no significant change in expression.

‡ Promoter methylation (%) before the intervention.

§ Promoter methylation (%) after the intervention.

‖ DNA methylation difference. This was calculated by subtracting the promoter methylation (%) before the intervention from promoter methylation (%) after the intervention.

¶ http://www.ncbi.nlm.nih.gov/

** Mean value was significantly lower than that in healthy controls ($P < 0.01$).

S. G. J. van Breda et al.

mucosa of the adenoma polyp patients when compared with the healthy control subjects, which could contribute to the increased ODC1 activity in CRC. However, we found no evidence that halving (i.e. 75 g/d) or doubling (i.e. 300 g/d) the consumption of vegetables for 2 weeks had a detectable effect on the extent of methylation of the chosen genes in humans.

Acknowledgements

References

1. Steinmetz KA & Potter JD (1996) Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc* **96**, 1027–1039.
2. Strachan T & Read AP (1999) Human gene expression. In *Human Molecular Genetics,* Chapter 8, pp. 169–208 [T Strachan and AP Read, editors]. Oxford: BIOS Scientific Publisher Ltd.
3. Feinberg AP, Ohlsson R & Henikoff S (2006) The epigenetic progenitor origin of human cancer. *Nat Rev Genet* **7**, 21–33.
4. Jubb AM, Bell SM & Quirke P (2001) Methylation and colorectal cancer. *J Pathol* **195**, 111–134.
5. Cameron EE, Bachman KE, Myohanen S, et al. (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* **21**, 103–107.
6. van Breda SG, van Agen E, Engels LG, et al. (2004) Altered vegetable intake affects pivotal carcinogenesis pathways in colon mucosa from adenoma patients and controls. *Carcinogenesis* **25**, 2207–2216.
7. Belshaw NJ, Elliott GO, Williams EA, et al. (2004) Use of DNA from human stools to detect aberrant CpG island methylation of genes implicated in colorectal cancer. *Cancer Epidemiol Biomarkers Prev* **13**, 1495–1501.
8. Raizis AM, Schmitt F & Jost JP (1995) A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. *Anal Biochem* **226**, 161–166.
9. Klose RJ & Bird AP (2006) Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* **31**, 89–97.
10. Mathers JC (2006) Candidate mechanisms for interactions between nutrients and genes. In *Nutrient-gene Interactions in Cancer*, pp. 19–36 [S-W Choi and F Simonetta, editors]. Boca Raton, FL: Taylor & Francis.
11. Christman JK, Sheikhnejad G, Dizik M, et al. (1993) Reversibility of changes in nucleic acid methylation and gene expression induced in rat liver by severe dietary methyl deficiency. *Carcinogenesis* **14**, 551–557.
12. Wainfan E & Poirier LA (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res* **52**, Suppl. 7, 2071s–2077s.
13. Martinez ME, O'Brien TG, Fultz KE, et al. (2003) Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc Natl Acad Sci U S A* **100**, 7859–7864.
14. Alhonen-Hongisto L, Leinonen P, Sinervirta R, et al. (1987) Mouse and human ornithine decarboxylase genes. Methylation polymorphism and amplification. *Biochem J* **242**, 205–210.
15. Halmekyto M, Hirvonen A, Wahlfors J, et al. (1989) Methylation of human ornithine decarboxylase gene before transfection abolishes its transient expression in Chinese hamster ovary cells. *Biochem Biophys Res Commun* **162**, 528–534.
16. Wahlfors J (1991) Certain changes in ornithine decarboxylase gene methylation accompany gene amplification. *Biochem J* **279**, 435–440.
17. Lawrance IC, Fiocchi C & Chakravarti S (2001) Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Hum Mol Genet* **10**, 445–456.
18. Cutfield WS, Hofman PL & Mitchell M, et al. (2007) Could epigenetics play a role in the developmental origins of health and disease? *Pediatr Res* **61**, 68R–75R.
19. Feil R (2006) Environmental and nutritional effects on the epigenetic regulation of genes. *Mutat Res* **600**, 46–57.
20. Mathers JC (2005) Reversal of DNA hypomethylation by folic acid supplements: possible role in colorectal cancer prevention. *Gut* **54**, 579–581.
21. Fang M, Chen D & Yang CS (2007) Dietary polyphenols may affect DNA methylation. *J Nutr* **137**, Suppl. 1, 223S–228S.