Title Page

DNA methylation of *ESR-1* and *N-33* in colorectal mucosa of patients with Ulcerative Colitis (UC)

Arasaradnam RP, PhD MRCP[1,2,3], Khoo KTJ[1,2] MS MRCS, Bradburn M[2] MD FRCS, Mathers JC[1] PhD and Kelly SB[2] MD FRCS

[1] Human Nutrition Research Centre, Newcastle University, Newcastle NE2 4HH, UK [2] Northumbria Healthcare NHS Trust, UK [3]Clinical Sciences Research Institute, University of Warwick, Coventry CV2 2DX, UK.

Correspondence to: Dr R P Arasaradnam.
Clinical Sciences Research Institute,
Medical School, University of Warwick,
Clifford Bridge Road, Coventry CV2 2DX
United Kingdom
Tel: 024 7696087
Fax: 024 76966090
E mail: r.arasaradnam@warwick.ac.uk

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ABSTRACT

Introduction: Epigenetic marking such as DNA methylation influence gene transcription and chromosomal stability and may also be affected by environmental exposures. Few studies exist on alteration in DNA methylation profiles (genomic and gene specific methylation) in patients with Ulcerative Colitis (UC) and none assessing its relationship with lifestyle exposures.

Aims & Methods: To assess genomic methylation and promoter methylation of the *ESR-1* (oestrogen receptor - 1) and *N-33* (tumour suppressor candidate-3) genes in the macroscopically normal mucosa of UC patients as well as to investigate effects of anthropometric and lifestyle exposures on DNA methylation. Sixty eight subjects were recruited (24 UC and 44 age and sex matched controls). Colorectal mucosal biopsies were obtained and DNA was extracted. Genomic DNA methylation was quantified using the tritium-labelled cytosine extension assay (^{3}[H] dCTP) whilst gene specific methylation was quantified using the COBRA method.

Results: The methylation level of both *ESR-1* and *N-33* genes were significantly higher in UC subjects compared with controls (7.9% vs 5.9%; $p = 0.015$ and 66% vs 9.3%; $p < 0.001$ respectively). There was no detectable difference in global DNA methylation between patients with UC and age and sex matched controls. No associations between indices of DNA methylation and anthropometric measures or smoking patterns were detected.

Conclusions: For the first time, we have shown increased methylation in the promoter regions of the putative tumour suppressor gene *N-33* in macroscopically normal mucosa of patients with UC. In addition, we have confirmed that methylation of *ESR-1* promoter is higher in UC patients compared with age and sex matched controls. These findings suggests that, inactivation through methylation of the putative tumour suppressor genes *N-33* and *ESR-1*, may not be associated with colorectal carcinogenesis in UC.

Key Words: DNA methylation, tumour suppressor genes, *ESR-1*, *N-33*, diet

Introduction

Epigenetic alterations (changes in genomic marks which are associated with altered gene expression but without changes in the DNA sequence) such as DNA methylation, are among the most common molecular alterations in human cancers including colorectal cancer (CRC)[1]. The addition of a methyl group to the carbon-5 position of cytosine is the only common covalent modification of human DNA[2]. This epigenetic modification occurs almost exclusively at cytosines that are followed immediately by guanine (CpG dinucleotides). CpG dinucleotides are under represented in the genome and those that are present are nearly always methylated. However, small stretches of DNA known as *CpG islands* (CGI; often located within the promoter regions of human genes), whilst rich in CpG dinucleotides, are nearly always methylation-free. Methylation of these CGI is associated frequently with transcriptional inactivation of the corresponding gene[3]. The methylation patterns are controlled by DNA methyltransferases (DNMT) as well as CpG methyl binding proteins (MBD)[3,4].

Genomic DNA hypomethylation has been implicated in the multi-step progression of colorectal carcinogenesis through a number of pathways including chromosomal instability, loss of imprinting, activation of oncogenes and microsatellite instability[5]. Correspondingly, hypermethylation of CpG islands is thought to occur in a non-stochastic manner. Such patterns of gene specific methylation may be useful as putative biomarkers of risk of CRC[6] as well as other age-related intestinal diseases. Methylation of specific genes has been shown to induce gene silencing and chromosomal instability through a number of pathways including inactivation of tumour suppressor genes, possibly

chromatin condensation and suppression of DNA repair mechanisms[7]. These same processes have been reported to occur during carcinogenesis[8]. For example, *p16*^{INK4a} a tumour suppressor gene which is inactivated in most human cancers, including CRC, has been shown to be silenced as a result of promoter methylation[9]. Similarly *ESR-1* (oestrogen receptor – 1) and *N-33* (tumour suppressor candidate -3), both putative tumour suppressor genes, have been shown to be hypermethylated in CRC[10] and ageing (an important risk for CRC)[11].

The promoter region of the *ESR-1* gene, a putative tumour suppressor, has been shown to be hypermethylated in both colon cancer as well as UC but few studies exist on gene specific (tumour suppressor) methylation in UC. Two small studies[12,13] have shown higher percentage methylation of *ESR-1* in neoplastic epithelium of UC patients compared with non-neoplastic tissue but were limited to small sample size and lack of a healthy control group for comparison[13]. We hypothesise that the DNA methylation profile may be altered in UC, specifically in the promoter regions of putative tumour suppressor genes (*ESR-1* and *N-33*). We investigated the relationship (if any) with lifestyle factors such as smoking and anthropometric measures.

Material and Methods

This case-control study was performed in Northumbria Healthcare NHS Foundation Trust, England. Ethical approval was obtained from the Northumbria Local Research Ethics Committee (Ref: 04/Q0902/61) and Research Governance approval was obtained from Northumbria Healthcare NHS Foundation Trust where patient recruitment was undertaken. A total of 68 patients (24 UC and 44 age and sex matched controls) were recruited. All 24 UC patients (18 male) had histological confirmation of disease. The validated Simple Colitis Clinical Activity Index (SCCAI) was used to assess disease activity[14]. Subject

characteristics including pharmacological therapy are summarised in Table 1. All UC patients had a colonoscopy in the preceding two years to assess disease extent. A three year follow up confirmed that none of the UC subjects had developed colorectal cancer. The control group (n = 44; 34 male, matched for age) had been referred to the endoscopy department at Wansbeck General Hospital, Northumbria Healthcare NHS Foundation Trust for a flexible sigmoidoscopy because of symptoms of either altered bowel habit or 'outlet type' rectal bleeding and were found to a have a normal colonic examination. For the purpose of this study, all patients underwent a flexible sigmoidoscopy to obtain rectal biopsies (disposable forceps; 10cm from the anal margin). Patients with macroscopic evidence of colonic inflammation, colonic adnenomas or colorectal cancer were excluded from the study.

The mucosal samples weighed 3-5 mg and DNA was extracted as per the manufacturer's protocol (QIAGEN, QIAmp® DNA mini kit). Proteinase K was added and samples were heated to 56°C overnight to ensure complete tissue lysis. Additional RNAase treatment was carried out to ensure RNA free DNA samples. Only samples which had an A260/280 wavelength (Eppendorf® Biophotometer) ratio greater than 1.7 were utilised.
The cytosine extension assay was used for the determination of global methylation status[15]. The assay uses the methylation sensitive restriction enzyme HPA 11 (New England Biolabs) to cut genomic DNA at its recognition sequence (CCGG) only if the CpG dinucleotide is unmethylated; methylated sections of the genome are protected from the cutting action of the enzyme. The resultant cutting leaves an overhanging 5' guanine residue, which is paired with a tritium labelled cytosine (3[H]dCTP) during the assay. The amount of radiation incorporated into the DNA during the assay is directly proportional to the number of cut and therefore unmethylated sites.
DNA methylation at specific gene loci was quantified by the COBRA technique (Combined Bisulphite Modified Restriction Enzyme Analysis) described by Xiong et al (1997)[16] in which DNA was treated with sodium

bisulphite followed by PCR and restriction enzyme digestion. The genomic region of interest was PCR amplified using primer sets designed specifically to contain no CpG dinucleotides. This step ensured that all templates were amplified equally regardless of the methylation status of the original genomic sequence. Following digestion with restriction enzymes separation of the digestion products using agarose gel electrophoresis, the DNA bands were visualised using Sybr green staining and the bands representing methylated and unmethylated sequences quantified using UVI software (UVI Tech Ltd.). In this study, the loci of interest were in the promoter regions of the *ESR-1* and *N-33* genes. Details of primer sets, restriction enzyme used and product sizes are given in Table 2.

Statistical Analysis

SPSS v15 was used for all data analysis. Genomic DNA methylation and methylation level for each gene (*ESR-1* and *N-33*) were used as the dependant variables. Data for genomic DNA methylation was normalised by transformation using Log base 10. Analysis of variance (ANOVA) was employed to analyse differences in genomic DNA methylation between UC and controls.

For the purpose of statistical analysis, *ESR*-1 and *N*-33 methylation were categorised binomially i.e. those samples with no detectable methylation (zero value) and those with detectable methylation (given a value of 1). Fisher's exact test was used to compare methylation between patient groups where the dependant variable was in binomial form. Binary logistic regression was employed to determine the relationship of anthropometric measures and smoking pattern with dependant variables (promoter methylation of *ESR*-1 and *N*-33). No outliers were identified. Statistical significance was defined as $p \leq 0.05$.

Results

ESR-1

There was a wide range in detectable CpG methylation of *ESR-1* in UC subjects compared with age and sex matched controls. The methylation level of *ESR-1* in UC subjects was 7.9% compared with 5.9% in controls ($p = 0.015$; χ^2 /Fisher's exact test) - Figure 1. Although the methylation level of *ESR-1* was higher in females compared with males in both UC (19% vs 10%) and controls (25% vs 5.1%), this difference was not significant. No significant association was noted with anthropometric measures or smoking pattern.

N-33

Whilst there was a wide range in detectable CpG methylation of *N-33* in both UC subjects and controls, the range was smaller than that observed for *ESR-1*. The mean detectable methylation of *N-33* in UC subjects (66%) was significantly higher than age and sex matched controls (9.3%) ($P = 0.001$; χ^2/Fisher's exact test) - Figure 2. Of note, methylation of *N-33* was detectable in all UC subjects. No significant difference in methylation of *N-33* was noted between males and females. As with *ESR-1*, *N*-33 promoter methylation was not significantly associated with anthropometric measures or smoking pattern.

Whilst all 24 UC subjects were *N-33* positive, only 38% were *ESR-1* positive. Similar proportions (38%) were found to be both *ESR-1* and *N-33* positive. In 63% of UC patients, *N-33* was positive whilst *ESR-1* was negative i.e. no detectable CpG methylation.

There was no relationship between the age at diagnosis, disease duration, number of flare ups, SCCAI score or disease extent and promoter methylation for either *ESR-1* or *N-33* genes.

Genomic DNA methylation

The geometric mean for genomic DNA methylation (quantified as ^{3}[H] dCTP incorporation into DNA: dpm/µg) was 3.6 (CI: 2.5 – 4.3) (10^{3} dpm/µg) for UC subjects compared with 4.1 (CI: 3.2 – 4.7) (10^{3} dpm/µg) for controls – Figure 3. A trend towards statistical significance was noted (p = 0.06).

Discussion

Ulcerative colitis is a chronic inflammatory condition with an increased risk of developing colorectal cancer[17,18] especially, in those diagnosed at a younger age and with greater extent of disease[19]. Several mechanisms have been proposed including genomic instability which is thought to explain the observed CRC risk associated with UC. Examples of molecular determinants used to assess the risk of CRC in UC include cellular proliferation, genomic DNA methylation[20] and microsatellite instability[21] amongst others.

Little is known about the mucosal inflammatory effect of UC on gene specific methylation. Using the COBRA assay, Issa and colleagues[12] found higher methylation of *ESR-1* in 12 UC subjects with high grade dysplasia or cancer compared with 6 UC patients without dysplasia. In that study, methylation levels were comparable for UC patients without dysplasia and healthy controls (n = 5). A later study by Tominaga et al (2005)[13], also using the COBRA method, reported higher levels of *ESR-1* methylation in the neoplastic epithelium of UC patients compared with non-neoplastic tissue (25% vs 4% respectively). There were also higher levels of methylation in the distal colon compared with the proximal colon but only in UC patients with neoplasia. No healthy controls were used. See Table 3 summarises the studies on gene specific methylation in UC.

In this study, both *ESR-1* and *N-33* promoter gene methylation levels were significantly higher in the macroscopically normal epithelium of UC subjects compared with age and sex matched controls. There was no evidence of dysplasia (confirmed histologically) and importantly on subsequent follow up at three years, no cancers were identified. This is in contrast to the study by Issa et al.[12] where there was no difference in *ESR-1* methylation between controls (n = 5) and UC subjects without dysplasia (n = 6). This lack of a difference may be explained by the relatively small sample size (only 6 UC patients without dysplasia and 5 controls) compared with this study which recruited 68 patients (24 UC subjects and 44 age and sex matched controls). Furthermore, the clinical characteristics of the UC subjects in the study by Issa et al.[12] were not described in detail. For example, it is not known whether the UC subjects without dysplasia had less active, less extensive disease and shorter duration of disease compared with those with dysplasia. In our study, we found no relationship between age at diagnosis, disease duration, number of flare-ups, SCCAI score or disease extent with promoter methylation of *ESR-1* or *N-33*.

We also found no statistical significant difference in genomic DNA methylation in UC patients compared with age and sex matched controls. This finding is in contrast with the only other report of genomic hypomethylation in UC patients[20]. These authors reported hypomethylation in 26 patients compared with 11 controls using the methyl acceptance assay which has several limitations (this assay is indirect, semi-quantitative and prone to false positives due to damaged DNA templates)[23]. The authors speculated that low levels of S-Adenosyl Methionine (SAM), a universal methyl donor, in active UC coupled with increased cell proliferation may impair DNA methylation status. In our study, although folate status was lower in UC subjects (data not shown), it was still within the normal reference range.

Our findings of CpG hypermethylation of the putative tumour suppressor genes *N-33* and *ESR-1* in normal tissue suggests that inactivation through methylation of these putative tumour suppressor genes may not be associated with development of CRC in UC patients. The functional role of *N-33* in UC remains unknown. This is the first study to our knowledge that has reported methylation of *N-33* within a cohort of patients with UC. It is plausible that *N-33* methylation in UC subjects may accelerate tissue ageing or perhaps even telomere shortening, which, has been reported in UC[24]. Further evaluation to measure *N-33* expression (at mRNA and protein level) as well as to confirm our findings in larger UC cohorts including those with dysplasia and UC associated cancer are required. Specifically, its association with habitual dietary intake – the latter being an important variable given the importance of diet on DNA methylation[25].

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Figure Legends

Figure 1: Promoter methylation level of *ESR-1* in UC subjects and controls.

Figure 2: Promoter methylation level of *N-33* in UC subjects and controls.

Figure 3: Genomic DNA methylation in UC subjects and controls.