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Smoking is associated with hypermethylation of the *APC* 1A promoter in colorectal cancer: the ColoCare Study

Timothy M. Barrow[1,2,3,†], **Hagen Klett**[1,3,4,†], **Reka Toth**[1,5], **Jürgen Böhm**[1,5], **Biljana Gigic**[1,5,6], **Nina Habermann**[1,5], **Dominique Scherer**[1,5,7], **Petra Schrotz-King**[1,5], **Stephanie Skender**[1,5], **Clare Abbenhardt-Martin**[1,5], **Lin Zielske**[1,5], **Martin Schneider**[6], **Alexis Ulrich**[6], **Peter Schirmacher**[1,8], **Esther Herpel**[1,8], **Hermann Brenner**[1,5,9], **Hauke Busch**[4,12,#], **Melanie Boerries**[1,3,4,#], **Cornelia M. Ulrich**[1,5,10,#], and **Karin B. Michels**[1,2,11,*,#]

[1]German Cancer Consortium (DKTK), Heidelberg, Germany

[2]Institute for Prevention and Cancer Epidemiology, Faculty of Medicine and Medical Center, University of Freiburg, Freiburg, Germany

[3]German Cancer Research Center (DKFZ), Heidelberg, Germany

[4]Institute of Molecular Medicine and Cell Research, Faculty of Medicine and Medical Center, University of Freiburg, Freiburg, Germany

[5]Division of Preventive Oncology, National Center for Tumor Diseases (NCT) and German Cancer Research Center (DKFZ), Heidelberg, Germany

[6]Department of Surgical Oncology, University Clinic Heidelberg, Heidelberg, Germany

[7]Institute of Medical Biometry and Informatics, University of Heidelberg, Heidelberg, Germany

[8]Department of General Pathology, University Clinic Heidelberg, Heidelberg, Germany

[9]Division of Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Heidelberg, Germany

[10]Population Sciences, Huntsman Cancer Institute, Salt Lake City, Utah, USA

[11]Department of Epidemiology, UCLA Fielding School of Public Health, Los Angeles, CA, USA

[12]Lübeck Institute of Experimental Dermatology and Institute of Cardiogenetics, University of Lübeck, Lübeck, Germany

Abstract

[*]Correspondence to: Prof. Karin Michels, Institute for Prevention and Cancer Epidemiology, Faculty of Medicine and Medical Center, University of Freiburg, Freiburg, Germany. Tel: +49 270 77360, Fax: +49 270 77340, tumorepidemiologie@uniklinik-freiburg.de.
[†]These authors contributed equally to the work (First authors)
[#]These authors contributed equally to the work (Last authors)

Author contributions statement

CU conceived the cohort study. TB, RT, NH, CU and KM conceived the investigation into smoking. JB, LZ, MS, AU, PS and EH organised and performed the sample collection. RT, BG, DS, SS, CAM, PSK and H Brenner were involved in data collection and organisation. TB, HK, RT, H Busch and MB analysed the DNA methylation data. TB, HK, RT, CU and KM performed data interpretation. TB wrote the manuscript, with figures generated by TB and HK. All authors were involved in writing and had final approval of the submitted manuscript.

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Smoking tobacco is a known risk factor for the development of colorectal cancer, and for mortality associated with the disease. Smoking has been reported to be associated with changes in DNA methylation in blood and in lung tumour tissues, although there has been scant investigation of how epigenetic factors may be implicated in the increased risk of developing colorectal cancer. To identify epigenetic changes associated with smoking behaviours, we performed epigenome-wide analysis of DNA methylation in colorectal tumours from 36 never smokers, 47 former smokers and 13 active smokers, and adjacent mucosa from 49 never smokers, 64 former smokers and 18 active smokers. Our analyses identified 15 CpG sites within the *APC* 1A promoter that were significantly hypermethylated and 14 CpG loci within the *NFATC1* gene body that were significantly hypomethylated ($pLIS<1\times10^{-5}$) in tumours of active smokers. The *APC* 1A promoter was hypermethylated in 7 of 36 tumours from never smokers (19%), 12 of 47 tumours from former smokers (26%), and 8 of 13 tumours from active smokers (62%). Promoter hypermethylation was positively associated with duration of smoking (Spearman rank correlation, $\rho=0.26$, $p=0.03$) and was confined to tumours, with hypermethylation never being observed in adjacent mucosa. Further analysis of adjacent mucosa revealed significant hypomethylation of four loci associated with the *TNXB* gene in tissue from active smokers. Our findings provide exploratory evidence for hypermethylation of the key tumour suppressor gene *APC* being implicated in smoking-associated colorectal carcinogenesis. Further work is required to establish the validity of our observations in independent cohorts.

Keywords

Smoking; Tobacco; Colorectal cancer; Epigenetics; DNA methylation; APC

Introduction

Smoking tobacco is a risk factor for many forms of cancer, including colorectal cancer (CRC). Ever-smokers, which includes both current and former smokers, have an 18% increase in the risk of developing the disease relative to individuals who have never smoked [1], and the risk is greatest for the development of tumours in the rectum. In addition to increased incidence, active smokers have a 23% greater risk of CRC-related mortality [2] and patients who are former smokers still display increased risk of all-cause mortality [3]. The duration and intensity of smoking are known to modify risk, with individuals who have smoked for 30 years and those with 20 pack-years of smoking each displaying a 40% increase in risk of CRC-related mortality [3]. However, the mechanisms by which smoking tobacco increases CRC risk have not been elucidated. It has been hypothesised that the carcinogenic products of cigarette smoke may reach the colorectum through the blood, and are implicated in the early initiation of cancer, as opposed to furthering the development of existing adenomas [4].

Smoking is associated with alterations in DNA methylation, an epigenetic modifier of gene expression, in healthy individuals. Such epigenetic events display tissue-specificity [5] and differ by ethnicity [6,7], and can serve as markers of long-term exposure to tobacco smoke [8]. Several studies examining the blood of smokers have reported differential methylation of loci within the aryl hydrocarbon receptor repressor (*AHRR*) gene [6,9], a putative

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tumour-suppressor which mediates the detoxification of products in cigarette smoke, and the coagulation factor II (thrombin) receptor-like 3 (*F2RL3*) gene [6,8–10], implicated in blood clotting. Associations have been identified between smoking-related changes in DNA methylation of *AHRR*, *F2RL3* and LINE1 elements measured in blood and the risk of cancer [11] and mortality from the disease [12].

Further to these observations in healthy individuals there is evidence that smoking is associated with epigenetic changes in tumour tissue. Epigenome-wide association studies have identified distinct methylation profiles in lung tumours from smokers and non-smokers [13], and candidate-gene approaches have identified smoking-related changes in the methylation of cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16*) and runt-related transcription factor 3 (*RUNX3*) in bladder tumours [14,15] and *CDKN2A/p16* and O-6-methylguanine-DNA methyltransferase (*MGMT*) in lung tumours [16]. Smoking-related epigenetic events may occur early in carcinogenesis, as demonstrated by their observation in stage I non-small cell lung cancers [17]. However, the evidence for smoking-associated epigenetic dysregulation in CRC is currently limited. Smoking has been reported to be associated with microsatellite instability and positive CpG island methylator phenotype (CIMP) status [18], but there has otherwise been scant research into DNA methylation in colorectal tumours classified by smoking status.

In this study, we investigated whether epigenetic factors may be implicated in the increased risk of CRC among tobacco smokers by analysing epigenetic patterns in colorectal tumours and neighbouring mucosa in relation to smoking behaviours. We utilised the Illumina HumanMethylation450 microarray platform to analyse DNA methylation in samples taken from a total of 137 colorectal cancer patients, 51 of whom had never smoked ('never smokers'), 68 who had been smokers but had ceased at least two years prior to cancer diagnosis ('former smokers'), and 18 who smoked at the point of diagnosis ('active smokers'). We report that promoter 1A of the *APC* gene, commonly inactivated in CRC, is hypermethylated in the tumours of active smokers. Methylation of this region is associated with duration of smoking, and hypermethylation ($\beta>0.2$) was never observed in adjacent mucosa. Our results suggest that the increased risk of CRC development among smokers may progress through epigenetic inactivation of the key tumour suppressor gene *APC*.

Material and Methods

The ColoCare Study

The ColoCare consortium is a multicentre initiative of interdisciplinary research on outcomes associated with colorectal cancer, with sites at the Fred Hutchison Cancer Research Center (Seattle, USA), Moffit Cancer Center (Tampa, USA), and from 2010 at the German Cancer Research Center (Heidelberg, Germany). This study exclusively focussed upon patients recruited in Heidelberg. ColoCare has been approved by the ethics committee of the University of Heidelberg medical faculty. Patients were enrolled to this prospective cohort at the point of diagnosis, having given informed consent, with biospecimens and data collected at regularly scheduled intervals of 3, 6, 12, 24 and 36 months post-surgery. Medical factors were abstracted from patients' charts and records from the University Hospital of Heidelberg. Data on dietary habits, exercise and physical activity, smoking

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habits, medication, socio-demographic information, and quality of life were collected via questionnaires. To date, 500 patients have been recruited at the Heidelberg site.

Tissue samples

Tissue samples were collected from patients undergoing surgery at the University Hospital of Heidelberg, and were reviewed by pathologists to ensure their quality and origin. Tumour samples were collected from 36 patients who had never smoked, 47 who were former smokers, and 13 who were active smokers at the point of diagnosis. Mucosa was taken from adjacent to tumours from 49 never smokers, 64 former smokers and 18 active smokers. A summary of patient characteristics is provided in Table 1.

DNA isolation

DNA was extracted from fresh-frozen tissue using the QIAamp AllPrep DNA/RNA mini kit (Qiagen) according to the manufacturer's instructions.

Illumina Infinium HumanMethylation450 BeadChip microarrays

DNA microarrays were performed at the Genomics and Proteomics Core Facility at the German Cancer Research Center (Heidelberg, Germany). One μg of genomic DNA was bisulphite-converted using the EZ DNA Methylation kit (Zymo Research) according to the manufacturer's instructions. The microarray assays were then performed according to the Illumina Infinium HD Methylation protocol.

Microarray data analysis

Microarray data was pre-processed using the Illumina Genome Studio software program before analysis using the R minfi package. Background correction and dye-bias normalisation were performed using noob [19], and functional normalisation was performed to remove batch effects and inner technical variability and adjust for Type I/II probe fluorescence effect, as described elsewhere [20]. Prior to background correction and normalisation, probes with detection p values >0.01 in 10% of samples (n=662) or bead counts less than three in 10% of samples (n=162) were removed. Probes with SNPs within 10 bp of the target CpG with minor allele frequencies of >0.01 (n=19,099) and mapping to the X and Y chromosomes (n=11,150) were removed. Subsequently, a total of 456,144 probes were taken forward for analysis.

Loci that were differentially methylated by smoking behaviours were identified by fitting a linear least-squares regression model across the conditions followed by computing moderated t-statistics for every CpG site, as described in the limma pipeline [21]. Due to the non-independent structure of the univariate t-statistics, we used a non-homogenous hidden Markov model (NHMM) to incorporate the dependence coming from the chromosomal positions of CpGs in the test statistics, as proposed and described elsewhere [22]. In brief, t-statistics were z-score transformed and distances (base pairs) between CpGs calculated and used as dependence structure in the NHMM. The NHMM parameters were estimated by expectation maximisation with randomised initial values. To avoid local maxima in the maximisation algorithm we used 30 initialisations and chose the initialisation with the smallest Bayesian information criteria (BIC). This provides a reproducible local index of

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significance (LIS), as previously defined [23], and can be interpreted as dependence corrected p value (pLIS). For computational efficiency we performed the analysis by chromosome and pooled the results afterwards, with significance defined as pLIS$<1\times10^{-5}$. The pLIS scores were computed using the R package NHMMfdr. Comparisons were made between never smokers and active smokers and between never smokers and former smokers in tumour and adjacent mucosa tissues. To identify loci that are differentially methylated between tumours and adjacent mucosa in a smoking-specific manner, we compared the differences in active smokers of tumour and mucosa with the differences among never smokers of tumour and mucosa. All analyses were adjusted for age and sex in the linear regression model.

The methylation microarray dataset is available from the NCBI Gene Expression Omnibus repository (accession number: GSE101764).

Identification of probe-associated SNPs

To account for false positives stemming from genetic variation, we used the UCSC Genome Browser and NCBI dbSNP databases [24,25] to identify single nucleotide polymorphisms (SNPs) within the 50-mer probes of the microarray for sites identified as significantly differentially methylated by smoking behaviours. The unconverted DNA sequences (‘SourceSeq’) for each significantly-different probe in tumour tissue and adjacent mucosa were extracted from the GenomeStudio output file and were used to perform a BLAT search using the UCSC Genome Browser [24]. The minor allele frequencies for all SNPs located within the probe sequences were identified using the UCSC Genome Browser and the NCBI dbSNP database [24,25]. Data from across all ethnicities or, where available, European populations was recorded, using estimates from studies with the largest sample sizes.

Statistical analyses

Associations between DNA methylation and smoking habits were calculated using data on pack-years and duration of smoking for each patient, and time since cessation among former smokers. Detailed data on smoking habits was available for 87 patients from whom tumour tissue was taken and 115 patients providing adjacent mucosa. Associations between DNA methylation (beta values) and intensity (pack-years) and duration (years) of smoking were identified using Spearman’s rank correlation coefficient, as were associations with time since cessation of smoking (years). Associations between tumour location and *APC* promoter 1A hypermethylation were calculated using Fisher’s exact test. Statistical significance was defined as $p<0.05$.

Results

Characteristics of the patients

Details of the CRC patients from whom samples of colorectal tumours and adjacent mucosa were obtained are provided in Table 1. Tumour tissue was obtained from 36 never smokers, 47 former smokers and 13 active smokers, while adjacent mucosa was taken from 49 patients who were never smokers, 64 who were former smokers and 18 active smokers. Matched pairs of tumour and adjacent mucosa tissue were available for 89 of the patients (33

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never smokers, 43 former smokers and 13 active smokers). The mean level of smoking was 18.7 pack-years among active smokers and 12.7 pack-years among former smokers. The mean duration of smoking was 37.6 years among active smokers and 19.6 years among former smokers.

The *APC* promoter 1A is hypermethylated in the tumours of active smokers

Epigenome-wide analysis of DNA methylation in 96 colorectal tumours and 131 samples of adjacent mucosa was performed using the Illumina Infinium HumanMethylation450 BeadChip microarray platform at the German Cancer Research Center Genomics and Proteomics Core Facility (Heidelberg, Germany). An overview of performed analyses with the different comparisons is shown in Figure 1.

We identified 21 CpG sites where methylation was significantly different between tumours from patients who had never smoked and those who were active smokers at the point of diagnosis (Figure 2A). These mapped to 14 loci within the *NFATC1* gene, 6 within the *APC* gene, and 1 within *LAMB1* (Table 2). The 14 loci that mapped to the *NFATC1* gene were distributed throughout the gene body and predominantly located in CpG islands. In contrast, each of the six loci associated with *APC* corresponded to the 1A promoter region and were within a span of 83 bp. Median beta values at each of the six CpG sites were 0.41–0.53 higher in active smokers in comparison to never smokers. No CpG sites were differentially methylated between tumours from former smokers and never smokers.

Smoking-specific differential methylation between tumours and adjacent mucosa

We performed further analysis to identify genes that may be implicated in smoking-associated carcinogenesis by identifying loci that are differentially methylated between tumours and adjacent mucosa among active smokers but not never smokers. We identified 148 loci that were significantly differentially methylated between these conditions (Figure 2B, supplementary material, Table S1). This included all six of the loci previously identified within the *APC* 1A promoter and 9 of the 14 sites previously identified within the *NFATC1* gene body. The nine sites with greatest statistical significance all mapped to the *APC* 1A promoter, and a further six significantly differentially methylated sites were also identified within this region. The average beta values in tumours and adjacent mucosa from active smokers differed by >0.24 at each of the 15 sites of the *APC* 1A promoter, while differing by <0.10 in the same tissues from never smokers. Other genes prominently identified by this analysis included receptor-type tyrosine-protein phosphatase N2 (*PTPRN2*) and sidekick cell adhesion molecule 1 (*SDK1*).

APC promoter 1A methylation and tumour pathology

Our epigenome-wide analysis identified the *APC* promoter 1A as the leading target for smoking-associated methylation changes. This was confirmed by cross-validation analysis, which identified this region as the most predictive to distinguish between tumours from never and active smokers (supplementary material, Figure S1). We sought to further characterise methylation of this region by tumour pathology and smoking behaviours. Expanded analysis across the 15 significantly differentially methylated loci mapping to the *APC* 1A promoter revealed distinct hypermethylation in some patients (Figure 3A).

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Defining hypermethylation as mean beta values of >0.2, in accordance with our observed values across all tumours, the *APC* 1A promoter was hypermethylated in 7 of 36 tumours from never smokers (19%), 12 of 47 tumours from former smokers (26%), and 8 of 13 tumours from active smokers (62%). Across all smoking behaviours, hypermethylation was observed at all AJCC stages, including 4 of 8 stage I tumours (Figure 3B), and was more common in tumours located in the rectum (14 of 38 tumours, 37%) and distal colon (8 of 25, 32%) than in the proximal colon (2 of 14, 14%), but not significantly so (Fisher's exact test, p=0.18 and p=0.28 respectively). We identified no associations between methylation at the six differentially methylated loci within the *APC* 1A promoter and alcohol consumption (grams/day) or BMI (both $p > 0.05$). Hypermethylation of the 1A promoter was significantly more frequent among women (Fisher's exact test, p=0.02) and was associated with younger age (Spearman rank correlation, $\rho=-0.28$, p=0.01).

Methylation of the *APC* 1A promoter is associated with duration of smoking

To explore the relation between the intensity and duration of smoking with methylation of the *APC* 1A promoter, we utilised data for the 72 former and active smokers in this study regarding intensity (pack-years) and smoking duration (length of time for which the patient smoked). Additionally, for the 47 former smokers, the relation with the length of time between cessation of smoking and cancer diagnosis was also assessed. Greater duration of smoking was significantly and positively associated with increased methylation at cg14479889 ($\rho=0.27$, p=0.03) and trended towards significance at each of the other five differentially methylated loci ($\rho>0.19$, $p<0.09$) (Table 3). Most notably, the average methylation (beta values) across the 15 differentially methylated loci mapped to this promoter region was significantly and positively associated with duration of smoking ($\rho=0.26$, p=0.03). No significant associations were observed with pack-years of smoking ($p>0.29$) or time between cessation of smoking and cancer diagnosis among former smokers ($p>0.16$).

The *APC* promoter 1A is not hypermethylated in the mucosa adjacent to tumours

We examined *APC* promoter 1A methylation in mucosa adjacent to tumours, to determine whether hypermethylation of this region exists as a field defect. Matched tumour and adjacent mucosal tissue were available for 24 of the 27 patients with tumoural hypermethylation of the 1A promoter (irrespective of smoking status). No promoter hypermethylation was observed in the adjacent mucosa from any of the 24 patients (Figure 3C, average beta < 0.11), or individually at any of the six differentially methylated loci ($\beta<0.13$) (supplementary material, Figure S2).

TNXB is differentially methylated in the adjacent mucosa of smokers

To gain insight into how smoking may act upon the colon, such as through carcinogenic compounds from cigarette smoke carried in the blood or chronic inflammation, we performed epigenome-wide analyses of DNA methylation in adjacent mucosa by smoking behaviours. We identified four sites within a 500 bp region that map to the tenascin XB (*TNXB*) gene body that were significantly hypomethylated in mucosa from active smokers (supplementary material, Table S2). No differentially methylated loci were observed between former and never smokers.

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Discussion

In this study, we investigated how epigenetic factors may be implicated in conferring the increased risk of colorectal cancer among smokers by performing epigenome-wide analysis of DNA methylation in samples of tumours and adjacent mucosa by smoking behaviours. We report that smoking at the time of diagnosis is significantly associated with hypermethylation of the 1A promoter of *APC*, a key tumour suppressor gene that has been extensively studied with regard to colorectal cancer. Hypermethylation was unique to tumour tissue and was associated with the duration for which the patient has smoked. We observed that hypermethylation of this promoter was more common in the rectum and distal colon, in concordance with evidence that the association between smoking and CRC risk is greatest for developing tumours in the rectum [1,26]. Our findings may implicate the epigenetic silencing of *APC* in smoking-associated colorectal carcinogenesis. However, due to the relatively small number of patients who were active smokers at diagnosis, our results should be considered exploratory at this stage. We have been unable to validate our observations in an independent cohort due to the absence of publicly-available datasets incorporating smoking history, and insufficient numbers of active smokers at diagnosis within other studies. Further work in external cohorts is required to examine the validity of our observations.

APC is a tumour suppressor gene and regulator of the Wnt signalling pathway, which acts via regulation of β-catenin degradation and localisation. Loss of APC function has been proposed as a key early event in the development of sporadic colorectal cancer [27], with inactivation frequently occurring through mutations, especially in the mutation cluster region [28], and promoter methylation [29]. Expression of the 1A mRNA isoform of APC is regulated in part through methylation of promoter 1A (chr5:112,072,710–112,073,585) [30], and this region is aberrantly methylated in colorectal, breast and lung tumours, resulting in transcriptional silencing and increased activation of the Wnt signalling pathway [29,31]. We observed significantly greater methylation of this region in tumours from patients who were active smokers at the point of diagnosis, thereby linking smoking behaviours to silencing of this key tumour suppressor gene. It has been reported elsewhere that smoking is associated with mutations in *TP53* and *BRAF* but not *APC* [32], which together with our study may suggest that inactivation of this gene more commonly occurs through epigenetic dysregulation in smoking-associated CRC than through genetic changes. Median promoter methylation levels (beta values) were approximately 0.5 higher in active smokers (Figure 3), consistent with monoallelic methylation of the promoter. Although evidence from the mouse model suggests that inactivation of both alleles is required for tumourigenesis [33], monoallelic methylation of the *APC* promoter 1A is a frequent event in human colorectal tumours [31,34] and cancer cell lines [35], and has been reported in gastric tumours [36].

Interestingly, hypermethylation of the *APC* promoter 1A was never present in mucosa adjacent to the tumours. Methylation at each interrogated CpG site within promoter 1A was very highly conserved in adjacent mucosa, while in direct contrast there was substantial variation in promoter methylation between tumour samples (Figure 3C, supplementary material, Figure S2). Cancer is associated with significantly greater variability in DNA methylation than is found in healthy tissue, and this loss of stability and increased stochastic

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variation may facilitate malignant cells to adapt to changes in their microenvironments [37,38]. Genetic and epigenetic alterations implicated in carcinogenesis are sometimes present in the surrounding tissue as field defects [39,40], and increased variation in DNA methylation has been observed in cytologically-normal cells from individuals later diagnosed with cervical cancer [41]. However, we observed that methylation of the *APC* promoter 1A was still highly conserved in adjacent mucosa, in line with studies reporting an absence of *APC* hypermethylation in colonic mucosa [31]. Mutations in *APC* are sufficient to induce polyp formation in mice [42,43] and humans [44], and we therefore speculate that this absence of *APC* hypermethylation in adjacent mucosa may be due to the key role for loss of APC in driving carcinogenesis. Indeed, we observed hypermethylation of the 1A promoter in half of stage I tumours (Figure 3B). This hypothesis is further supported by evidence of *APC* promoter methylation being an early event in colorectal carcinogenesis that is detectable in small (<15 mm) adenomas [31].

We observed a significant association between promoter 1A hypermethylation and duration of smoking (Table 3), but further work is required to expand upon the relation between the intensity and duration of exposure and epigenetic events in CRC. Indeed, promoter hypermethylation was not observed in tumours from any of the 8 former smokers who had smoked for >35 years, and our epigenome-wide analysis did not identify any differentially methylated sites between never smokers and former smokers in tumours or adjacent mucosa. The cessation of smoking is known to reduce the risk of CIMP-high colorectal cancer and patients who quit >10 years prior to diagnosis display similar risk of CIMP-high tumours to never smokers [18]. Furthermore, it is known that methylation of the *AHRR* and *F2RL3* genes returns to normal levels with increasing time since cessation [9]. Therefore, as only 9 of the 40 former smokers in this study for whom there is relevant data ceased smoking <10 years prior to diagnosis, we speculate that the time since cessation may also be a significant factor in the risk of hypermethylation of *APC* promoter 1A.

Our epigenome-wide analysis also identified the *NFATC1* gene body as being hypomethylated in tumours from smokers (Figure 2). This gene encodes a transcription factor implicated in T cell activation. Epigenetic dysregulation of this gene has been observed in hepatocellular carcinoma [45] and lymphomas [46] while hypomethylation has been reported in healthy individuals with lower socioeconomic status [47]. Our study is the first to report hypomethylation of *NFATC1* in colorectal tumours. Overexpression of the gene is associated with worse prognosis in stage II and III colorectal cancer patients, which may occur through the promotion of cell migration and metastasis [48,49]. As the ColoCare Study began to recruit patients in October 2010, we are currently unable to determine whether *NFATC1* methylation is associated with patient prognosis in this cohort. We will be able to address this question in time as further data regarding patient outcomes is collected.

Our data suggests that smoking is not associated with the accumulation of widespread epigenetic defects in the adjacent mucosa. Methylation of the *APC* promoter 1A occurs independently of other epigenetic events in CRC [31], and we identified only one gene, *TNXB*, as differentially methylated in the adjacent mucosa of active smokers (Table 3). This may be considered to be in contrast to the findings of Paun *et al* [50], who reported disruption of normal gene methylation profiles in the normal rectal mucosa of smokers. We

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speculate that this may be the product of our analyses identifying genes implicated in malignant transformation due to our comparison of tumours and adjacent mucosa, while Paun *et al* examined rectal mucosa prior to the advent of tumour formation. To our knowledge, ours is the first study to observe differential methylation of *TNXB* by smoking behaviours. Further work is required to investigate how this extracellular matrix glycoprotein could be implicated in smoking-associated carcinogenesis.

Further to the inability to confirm our findings in an independent cohort, the comparatively low number of patients who actively smoked at the point of diagnosis is a limitation of this study, and one which could inhibit the identification of associations between smoking and methylation. We therefore incorporated the chromosomal position into test statistics by means of a NHMM, which also served to reduce the probability of secluded differentially methylated CpGs and hence most likely false positives. A particular strength of this study is the analysis of both tumour tissue and adjacent mucosa, which has enabled us to gain greater insight by identifying epigenetic events associated with smoking that are uniquely found in tumour tissue (hypermethylation of the *APC* promoter 1A) and to establish an absence of field defects associated with smoking in the neighbouring mucosa.

In conclusion, we report exploratory evidence for hypermethylation of the *APC* promoter 1A being implicated in the development of colorectal tumours among smokers. Methylation of this region was significantly associated with smoking at the point of diagnosis and with the duration of time for which the patient smoked, and hypermethylation was confined to tumours. Further work is required to validate our observations in independent cohorts, and to identify implications for patient prognosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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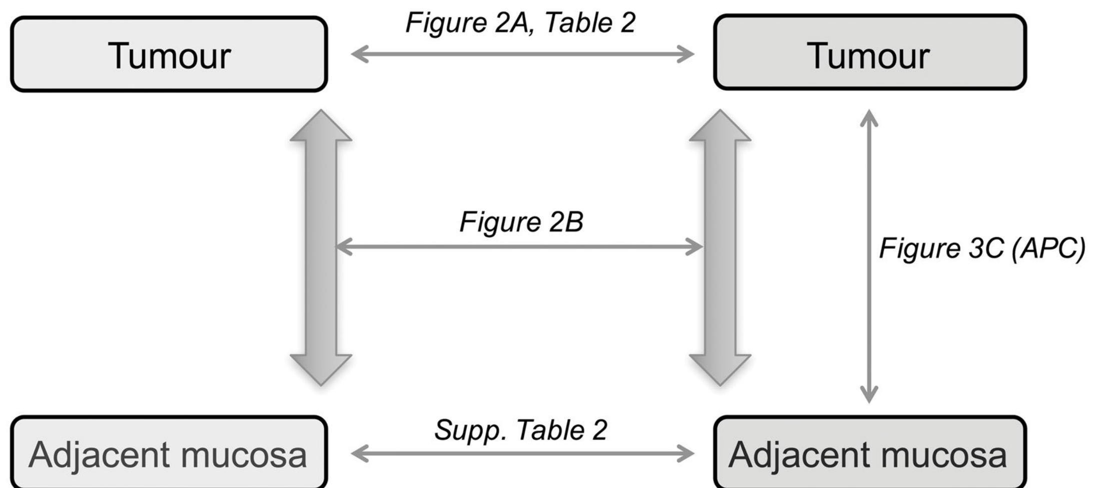

Figure 1.

Overview of analyses by smoking behaviours in tumours and adjacent mucosa. Differentially methylated sites between smokers and never smokers were identified in tumour tissue and in adjacent mucosa. Further analyses were performed to identify sites displaying smoking-specific differential methylation between tumours and adjacent mucosa.

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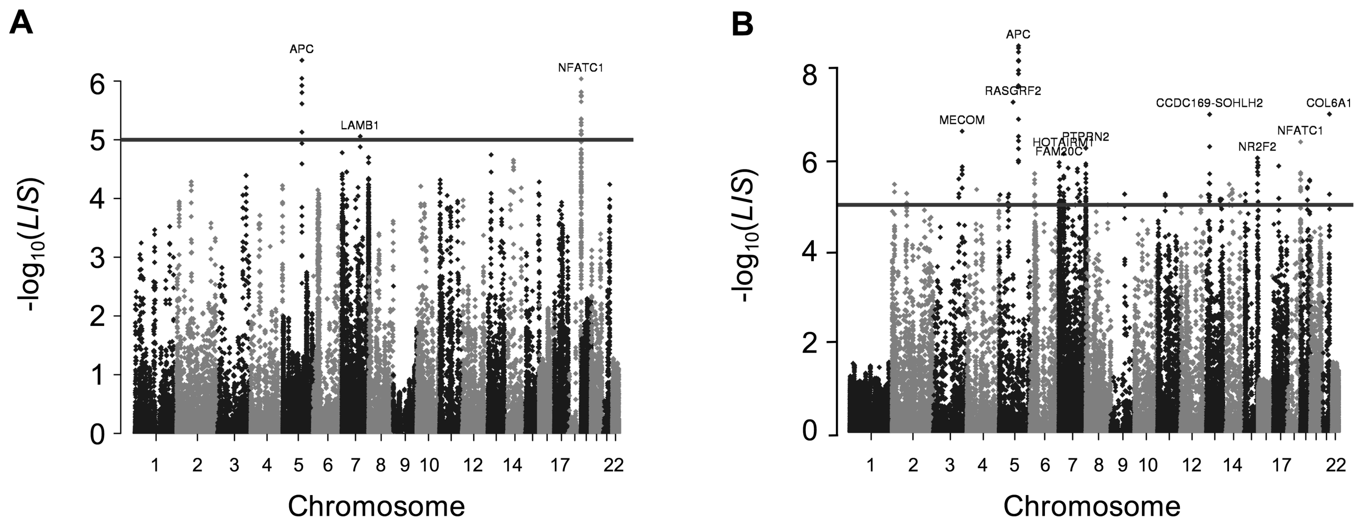

Figure 2.

Manhattan plots showing differentially methylated sites between never and active smokers. Results of the analyses between tumours from never and active smokers (A) and differential methylation between tumours and adjacent mucosa unique to active smokers (B). Gene symbols of the genes associated with the most significantly different sites are provided. The threshold (line) represents statistical significance (pLIS$<1\times10^{-5}$)

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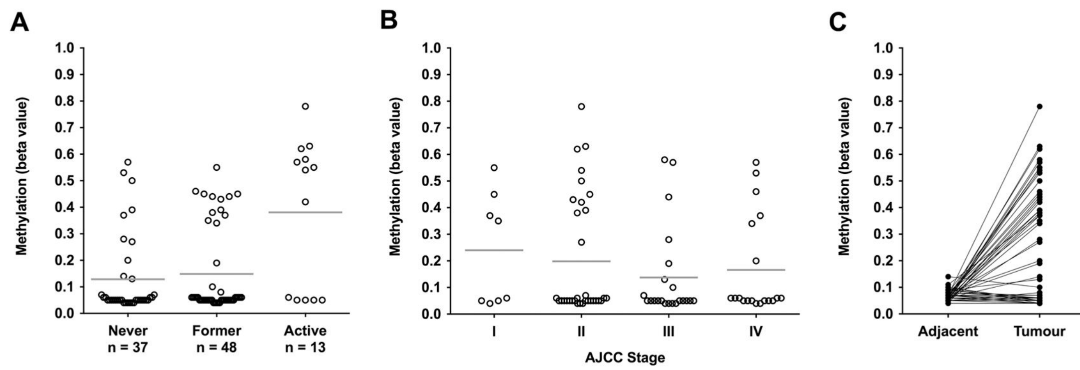

Figure 3.

Methylation of the *APC* promoter 1A in tumours and matched adjacent mucosa. Mean methylation levels (beta values) for each patient were calculated across the 15 CpG sites mapping to the 1A promoter that were identified as differentially methylated by smoking status (Figure 2). (A) promoter methylation in tumours by patient smoking status. Mean values by smoking status are indicated by horizontal lines. (B) promoter methylation in tumours by AJCC stage in all patients. Mean values by stage are indicated by horizontal lines. (C) promoter methylation in matched samples of tumours and adjacent mucosa from 89 patients (33 never smokers, 43 former smokers, and 13 active smokers). Lines indicate matched samples from the same patient.

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Table 1

Clinical and demographic characteristics of the patients.

		Adjacent mucosa			Tumour		
		Never	**Former**	**Active**	**Never**	**Former**	**Active**
Patients	***n***	49	64	18	36	47	13
Age	**Mean**	63.7	65.5	56.6	63.9	65.1	59.6
	SD	11.8	10.9	12.5	11.5	10.3	9.8
	Range	34 – 82	38 – 89	22 – 79	41 – 82	38 – 89	35 – 79
Gender	**Male**	24	48	10	21	37	6
	Female	25	16	8	15	10	7
Stage	**I**	8	8	4	3	5	1
	II	15	24	7	12	17	7
	III	13	18	5	12	14	4
	IV	11	14	2	9	11	1
Pack-years	**Mean (years)**	-	11.5	18.7	-	12.6	16.4
	0 – 9 (n)	-	31	5	-	22	4
	10 – 19 (n)	-	15	4	-	11	3
	20 (n)	-	11	6	-	11	4
Duration	**Mean (years)**	-	18.8	31.8	-	19.6	37.6
	0 – 9 (n)	-	18	2	-	11	0
	10 – 19 (n)	-	17	3	-	11	2
	20 – 29 (n)	-	13	2	-	8	0
	30 (n)	-	15	17	-	11	9

Data are provided regarding the age (mean, standard deviation, and range), gender, tumour stage and pack-years of smoking for the patients according to smoking status at the point of cancer diagnosis.

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Table 2

CpG sites with differential methylation by smoking status in tumours

Probe ID	Chromosomal location	Gene	Gene region	Island status	Mean β-value		LIS p value
					Never	Active	
cg08571859	chr5:112073350	APC	TSS1500	Open sea	0.11	0.36	7.4×10^{-6}
cg14511739	chr5:112073373	APC	TSS200	Open sea	0.11	0.39	1.2×10^{-6}
cg22035501	chr5:112073426	APC	TSS200	Open sea	0.12	0.42	4.4×10^{-7}
cg11613015	chr5:112073433	APC	TSS200	Open sea	0.10	0.34	9.0×10^{-7}
cg14479889	chr5:112073426	APC	TSS200	Open sea	0.12	0.38	1.6×10^{-6}
cg16970232	chr5:112073433	APC	TSS200	Open sea	0.13	0.40	2.4×10^{-6}
cg04744624	chr7: 107641770	LAMB1	Body	N_Shore	0.23	0.41	8.8×10^{-6}
cg15138382	chr18: 77186504	NFATC1	Body/5′ UTR	Island	0.89	0.75	1.8×10^{-6}
cg05302701	chr18: 77196320	NFATC1	Body/5′ UTR	Island	0.81	0.68	4.4×10^{-6}
cg18092363	chr18: 77202678	NFATC1	Body/5′ UTR	Island	0.94	0.85	7.9×10^{-6}
cg26550337	chr18: 77203542	NFATC1	Body/5′ UTR	Island	0.81	0.70	1.5×10^{-6}
cg26100137	chr18: 77203667	NFATC1	Body/5′ UTR	Island	0.97	0.90	2.3×10^{-6}
cg22279865	chr18: 77204561	NFATC1	Body/5′ UTR	S_Shore	0.93	0.87	4.5×10^{-6}
cg00445548	chr18: 77207209	NFATC1	Body/5′ UTR	Island	0.93	0.82	7.1×10^{-6}
cg02675550	chr18: 77208807	NFATC1	Body/5′ UTR	Island	0.86	0.75	5.6×10^{-6}
cg21242663	chr18: 77208881	NFATC1	Body	Island	0.90	0.81	8.1×10^{-6}
cg25595641	chr18: 77208991	NFATC1	Body	Island	0.94	0.86	1.8×10^{-6}
cg21806238	chr18: 77210990	NFATC1	Body	Island	0.92	0.84	5.1×10^{-6}
cg16253249	chr18: 77211212	NFATC1	Body	Island	0.81	0.74	1.8×10^{-6}
cg03239925	chr18: 77230795	NFATC1	Body	Island	0.74	0.63	7.0×10^{-6}
cg22324981	chr18: 77283493	NFATC1	Body	N_Shore	0.80	0.58	9.2×10^{-7}

Loci with significantly different methylation between tumours from never smokers and active smokers are listed, including Illumina annotation data. Median beta values are provided, along with pLIS values.

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Table 3

Associations between DNA methylation and smoking intensity and duration in tumours

Probe ID	Chromosomal location	Gene	Pack-years		Duration		Time since cessation	
			ρ	p	ρ	p	ρ	p
cg08571859	chr5:112073350	APC	−0.07	0.31	0.22	0.06	−0.05	0.41
cg14511739	chr5:112073373	APC	−0.01	0.47	0.19	0.09	−0.05	0.41
cg22035501	chr5:112073426	APC	−0.08	0.29	0.19	0.09	−0.11	0.31
cg11613015	chr5:112073433	APC	−0.02	0.44	0.20	0.08	−0.14	0.27
cg14479889	chr5:112073426	APC	−0.05	0.37	**0.27**	**0.03**	−0.21	0.17
cg16970232	chr5:112073433	APC	−0.01	0.48	0.21	0.07	−0.10	0.32
Promoter 1A	chr5:112,072,710 - 112,073,585	APC	0.02	0.44	**0.26**	**0.03**	−0.20	0.19

Spearman's rank correlation coefficients were calculated for each of the significantly different loci in tumour tissue, using data from former (n=47) and active (n=13) smokers. Correlations were calculated between methylation (beta values) and the pack-years of smoking or duration (years) of smoking. Additionally, for former smokers, correlations between methylation and time since cessation were calculated. ρ and p values are provided, with significant values highlighted in bold.