Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking

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A single cytosine–guanine dinucleotide (CpG) site within coagulation factor II (thrombin) receptor-like 3 (F2RL3) was recently found to be hypomethylated in peripheral blood genomic DNA from smokers compared with former and non-smokers. We performed two epigenome-wide association studies (EWAS) nested in a prospective healthy cohort using the Illumina 450K Methylation Beadchip. The two populations consisted of matched pairs of healthy individuals (n = 374), of which half went on to develop breast or colon cancer. The association was analysed between methylation and smoking status, as well as cancer risk. In addition to the same locus in F2RL3, we report several loci that are hypomethylated in smokers compared with former and non-smokers, including an intragenic region of the aryl hydrocarbon receptor repressor gene (AHRR; cg05575921, \( P = 2.31 \times 10^{-15} \); effect size = 14–17%), an intergenic CpG island on 2q37.1 (cg21566642, \( P = 3.73 \times 10^{-13} \); effect size = 12%) and a further intergenic region at 6p21.33 (cg06126421, \( P = 4.96 \times 10^{-11} \), effect size = 7–8%). Bisulphite pyrosequencing validated six loci in a further independent population of healthy individuals (n = 180). Methylation levels in AHRR were also significantly decreased (\( P < 0.001 \)) and expression increased (\( P = 0.0047 \)) in the lung tissue of current smokers compared with non-smokers. This was further validated in a mouse model of smoke exposure. We observed an association with breast cancer risk for the 2q37.1 locus (\( P = 0.003 \), adjusted for the smoking status), but not for the other loci associated with smoking. These data show that smoking has a direct effect on the epigenome in lung tissue, which is also detectable in peripheral blood DNA and may contribute to cancer risk.

INTRODUCTION

Epigenetic profiles, including methylation of the 5-carbon of cytosines, are helping us to unravel the pathogenesis of numerous complex diseases, in particular for diseases that have an environmental component to their aetiology. Since the first studies by Doll in the 1950s that linked smoking with lung cancer risk, smoking has been identified as a major risk factor for numerous cancers. However, the molecular changes that occur as a consequence of the neoplastic process itself, in addition to changes caused by chemo- and...
Radiotherapy, mean that any investigation into the epigenetic alterations induced by cancer risk factors must be performed prospectively in pre-diagnostic samples.

Improvements in the array-based technology have enabled the molecular effects of altered environments, such as cigarette smoke inhalation, to be assessed in larger populations. The only study to show a significant link between smoking and the epigenome used the Illumina 27K platform and identified a single locus in the coagulation factor II (thrombin) receptor-like 3 (F2RL3) gene out of 27,578 loci tested that was less methylated in current smokers (n = 65) compared with former (n = 56) and non-smokers (n = 56) (1). This gene is associated with platelet activation and coagulation, but cannot be clearly connected to the carcinogenic processes that are induced by an individual’s exposure to tobacco smoke.

The introduction of the Illumina 450K methylation bead array has enabled the analysis of the DNA methylation pattern across the genome with additional coverage of promoters, 5′ UTRs, first exons, gene bodies and 3′ UTRs. This study aimed to assess the impact of current and former smoking on DNA methylation in an epigenome-wide approach using prospectively collected blood samples from healthy individuals who subsequently developed breast or colon cancer compared with matched controls. We hypothesized that smoking induces gene-specific methylation, detectable in peripheral blood DNA, and that these exposure induced changes may impact on cancer risk.

**RESULTS**

**Epigenome-wide association studies**

In this study, we performed two epigenome-wide association studies (EWASs) on genomic DNA from peripheral white blood cells (WBCs) that were prospectively collected from two nested case–control studies within a large cohort from the general population. All individuals were healthy at the time of blood collection, but cases were selected from individuals who subsequently developed either breast or colon cancer (average lag-time to diagnosis = 4.6 and 7 years, respectively). These samples were being analysed to identify markers associated with breast cancer and colon cancer risks and survival; however, these analyses are under-powered to detect genome-wide significant individual markers and will need to be validated in larger sample sizes currently underway (see Supplementary Material, Figs S1 and S2). We have used these data in order to further examine the association between methylation in peripheral blood genomic DNA from smokers compared with former and non-smokers (1). For the present analysis, we used multivariate linear regression to investigate the association between DNA methylation levels and smoking status, adjusting for age and batch. Using a cutoff of P < 1 × 10⁻⁵, we identified 17 and 19 loci in the breast cancer and colon cancer EWAS, respectively, that were differentially methylated between smokers, former smokers and those who had never smoked (Fig. 1). Eight of these loci were shared by both studies (Table 1). The top hits for both studies (P < 1 × 10⁻⁵) are shown in Supplementary Material, Table S1. In all instances, the degree of methylation was lower in smokers than in non-smokers, and less difference was found between former smokers and non-smokers. Of note, the F2RL3 locus previously identified was in this list. There was no strong association (P < 1 × 10⁻⁵) between methylation levels at any of these loci and disease status, despite smoking being a weak risk factor for colon cancer (Supplementary Material, Table S2) (2,3). We observed no evidence for an association between smoking and the risk of colon cancer in this study (P = 0.857), which was likely to be a consequence of the small numbers of participants in the study. One of the smoking-associated loci (cg01940273) at 2q37.1 showed an association with developing breast cancer, after adjustment for smoking (P = 0.003) and estrogen receptor status (P = 0.035). This association showed significant heterogeneity by smoking status as it associates with the breast cancer case–control status in a logistic regression model (interaction, P = 0.039). This region, therefore, warrants further investigation in larger studies as a cancer risk marker and a mechanism for smoking-induced carcinogenesis.

**Bisulphite pyrosequencing validation**

We used bisulphite pyrosequencing on an additional set of healthy subjects (n = 180) to validate the methylation association with smoking using an alternative method. We validated the methylation of six cytosine–guanine dinucleotide (CpG) sites identified by the 450K array (two CpG sites in AHRR, two CpG sites in 2q37, one CpG site in F2RL3 and one CpG site in 6p21.33). The direction of methylation change and effect sizes were of the same magnitude in the validation groups, and again, a significant association with smoking was observed (Table 1). There was also remarkable evidence for an association between methylation levels and smoking intensity for the AHRR, 2q37 and 6p21 loci (P < 0.0005), but not F2RL3, with individuals smoking four or more cigarettes per day having significantly lower methylation levels at these genomic loci (Supplementary Material, Fig. S4 and Table S3). In former smokers, the methylation levels at these genomic loci returned to the levels of non-smokers with increasing time from cessation and those who had smoked more intensively had methylation levels that were closer to those of current smokers (Supplementary Material, Fig. S5).

Regional association plots showing the intragenic CpG island in AHRR and the intergenic CpG island at 2q37 are shown in Figure 2 with examples and pyrosequencing validation shown in Supplementary Material, Figure S6. The CpG site in one of the AHRR regions of interest (AHRR_p1, cg23576855) was also the site of a CG→CA SNP (rs6869832), with an A allele frequency of ~10% as confirmed by pyrosequencing (Fig. 3). Minor allele (A) carriers (n = 31) were excluded from the pyrosequencing statistical analysis, as the cytosine could not be methylated in the CpA dinucleotide. Interestingly, three of the current smokers in the validation set were heterozygous carriers; their methylation levels (mean, 37.7%) were approximately half of the value found in smokers who were homozygous for the G allele (mean, 66.9%; Table 1). This indicated that the CpG site on the G allele was methylated to a similar degree as homozygous G alleles. We did not have genotyping data on the individuals in the EWAS sample sets, but predict that
the individuals with $\sim 40$ and $5\% \beta$-values are likely to be heterozygous and homozygous for this single nucleotide polymorphism (SNP), respectively (Supplementary Material, Fig. S6).

**Methylation and expression in human and mouse lung tissue**

Pyrosequencing assays were conducted on bisulphite-converted genomic DNA extracted from 27 human lung samples. We have investigated lung tissue in this case to assess the most relevant tissue to the initial exposure. A marked association was present between the smoking status and methylation levels in human lung tissue. As in peripheral circulating mononuclear cell DNA, the methylation levels at cg23576855 and cg21161138 in the AHR gene were significantly decreased in current smokers ($P = 0.00327$ and $0.00143$, respectively) (Fig. 4A). The methylation values in lung tissue were also identical to those shown in peripheral blood mononuclear cells, which suggests that blood sampling could offer a useful surrogate for future biomarker studies of lung tissue methylation for this gene.

Expression analyses using qRT-PCR for AHR in human lung samples from smokers versus non-smokers ($n = 5$ for each group) was inversely correlated with methylation levels ($R^2 = 0.157$) and showed increased expression by 5.7-fold ($P = 0.0047$) in the current smokers compared with the non-smokers (Fig. 4B). In a mouse model of smoke exposure (4), smoking significantly increased the levels of Ahr and Cyp1a1 in a time-dependent manner compared with controls (Fig. 4C), while Ahr expression was initially reduced by 2.6-fold after the initial exposure to smoke (3 d; $P = 4.56 \times 10^{-6}$), but increased by 1.7-fold after 28 days ($P = 0.003$). Methylation of the Ahrr locus was not performed as this

**Figure 1.** Manhattan plot and quantile–quantile (QQ) plot for EWAS results for smoking status in two case–control studies. (A) Breast cancer case–control study. (B) Colon cancer case–control study. In the Manhattan plot, the vertical axis indicates (-log$_{10}$ transformed) observed $P$-values, and the horizontal thresholds indicate the significance levels ($P = 1 \times 10^{-5}$ and $P = 1 \times 10^{-3}$). In the QQ plot, the horizontal axis shows (-log$_{10}$ transformed) expected $P$-values, and the vertical axis indicates (-log$_{10}$ transformed) observed $P$-values. The lambda inflation factor (median[obs]/median[exp]) is shown.
Table 1. Leading differentially methylated genomic loci between smokers and former or non-smokers in a breast and colonic case–control cohort

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<th>Pyrosequencing methylation in the validation cohort</th>
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This list contains the top eight overlapping CG sites between the breast cancer and colon cancer EWAS studies, in addition to an additional site within the 2q37.1 locus that was also validated by pyrosequencing methylation in the validation cohort.

aLogistic regression adjusting for age and batch effect.

bEffect size represents percent methylation difference between current smokers and individuals who have never smoked. Pyrosequencing assays could not be designed for three of the nine CpG sites.

DISCUSSION

The findings from these three groups of individuals give strong evidence for the role of smoking in inducing changes in DNA methylation levels. In this study, we have validated previously identified associations with methylation and smoking for the F2RL3 and AHRR probes and have identified further AHRR probes that were significantly associated with smoking (1,5).

We have identified these in two cancer case–control studies using the Illumina 450K methylation beadchip and validated them by bisulphite pyrosequencing in an additional validation cohort. Our study has identified two novel loci, at 2q37.1 and 6p21, which are also strongly associated with the smoking status. Importantly, we show for the first time that one of these loci, 2q37.1, is also associated with breast cancer risk.

Lastly, we show that these associations between smoking and methylation are unlikely to be cell-type-specific differences due to different blood cell proportions.

Previous evidence has suggested that intragenic methylation levels are correlated with expression levels, with highly expressed genes having high levels of intragenic methylation, and vice versa (6–8). According to this rationale, we predicted that decreased levels of methylation in the AHRR gene in smokers would indicate lower levels of expression. In the smoke-exposed mice, this was indeed the case with short-term exposure (3 days), but the consequent increase in Ahrr expression after 28 days of smoke exposure suggests that other compensatory mechanisms of gene induction override the short-term decrease in expression marked by lower intragenic DNA methylation levels. This supports data that show an increase in Ahrr expression following exposure to benzo(a)pyrene, a chemical found in cigarette smoke, in a mouse model (9).

In humans, the expression of AHRR in lung tissue mirrored the long-term exposure in mice; however, the observed hypomethylation of this locus in lung tissue may be an indicator of past expression changes in this differentiated tissue type.

A recent study of 165 individuals on the 450K methylation array identified a single CpG site within the AHRR gene associated with the smoking status in EBV-transformed lymphoblastoid cell lines (5). With a larger study size of 554 individuals, we have validated the association and identified additional intragenic CpG sites in the AHRR gene associated with smoking in WBC DNA and lung tissue. The aryl hydrocarbon receptor (AHRR) is a crucial receptor in the pathway that metabolizes a range of biological compounds and synthetic environmental pollutants. Benzopyrene and dioxin-like compounds are highly toxic organic molecules that are released from various components of cigarettes during smoking and enter the circulation via the pulmonary vasculature. These compounds are metabolized by AhR (10,11), releasing further carcinogenic metabolites that have been implicated in lung cancer development (12). However, the AHRR pathway may also mediate carcinogenesis through other pathways such as oxidative stress (13). AHRR (aryl hydrocarbon receptor repressor; chromosome position 5p15.33) encodes for a class E basic helix–loop–helix intragenic CpG island region is not conserved in the mouse genome.
protein (14), which is mainly found in the cytoplasm. It inhibits the translocation of the AHR–ligand complex into the nucleus by disrupting the binding of AHR to the AHR-nuclear translocator (AHRNT) (15). The AHR–EAHRNT heterodimer also inhibits the transcription of AHRR in a feedback loop. The knockdown of AHRR has been shown to produce increased tumour cell invasiveness in a range of tissue types, including breast, colon, lung and ovarian (16,17). An increase of the repressor of the AHR pathway should lead to a decrease in the activity of the pathway, and therefore, a decrease in AHR pathway-mediated carcinogenesis. However, the data from our study show an increase in AHRR expression and decreased intragenic methylation due to smoking, with no evidence of an association with breast or colon cancer risk. Therefore, more work is needed to understand the complex mechanisms of smoking-induced carcinogenesis via the AHR pathway.

A novel finding of this study was that a genomic locus, comprising four consecutive probes at 2q37.1, was differentially methylated between smokers and former or non-smokers. The four probes are located within 824 bp of an intergenic CpG island (chr2: 233,284,112–233,284,935; http://genome.ucsc.edu/, hg19). This region maps to a DNase hypersensitivity site within a CpG island, indicating a possible regulatory region (Supplementary Material, Fig. S7) (18), and is a potential pseudogene of ECEL1 (endothelin-converting enzyme-like 1; chr2: 233,344,537 to 233,351,464 bp, 60,425 bp downstream of 2q37), as it possesses a high level of sequence homology (>95%). We observed an association with breast cancer risk at this locus with significant heterogeneity in the effect in smokers compared with non-smokers. While the estimate of breast cancer risk associated with smoking is still unclear (19), future work on larger case–control studies will be needed to validate this cancer association. We hypothesize that molecular markers of smoking exposure, such as
Smoking induces methylation and expression changes in human and mouse lung tissues. (A) Pyrosequencing data from two regions of interest, presented as boxplots, in human lung tissue samples from smokers ($n = 14$) versus never smokers ($n = 13$). For cg23576855 individuals heterozygous for the CG>CA polymorphism are marked as crosses. (B) AHRR mRNA expression data from the human lung tissue of smokers ($n = 5$) versus non-smokers ($n = 5$), showing a 5.7-fold increase in expression in smokers compared with non-smokers (upper panel) and correlation between methylation and expression in the same five non-smokers (grey circles) and five smokers (black triangles) (lower panel). (C) Mouse lung expression changes in AHRR and F2RL3, in addition to two other genes from the aryl hydrocarbon receptor pathway, AHR and CYP1A1. As predicted, AHR and CYP1A1 increase in response to cigarette smoke, and the expression of AHRR initially decreases after 3 days, before increasing after 28 days. There was no significant change in the expression of F2RL3 after exposure to cigarette smoke.
methylated markers described here, will provide more accurate measurement of the exposure than questionnaire-based data and allow a more robust assessment of any associated cancer risk. Whether this 2q37 locus is causally linked to breast cancer risk due to smoking is not yet known; however, if verified we hypothesize that the mechanism may involve in cis regulation of the developmentally regulated homologue, ECEL1. There are several limitations in establishing causality between an epigenetic trait, an exposure and cancer risk. These include the reversible nature of epigenetic modifications and the need for an appropriate tissue type in which to investigate gene expression—methylation associations (for example, a large epidemiology sized sample set of normal breast tissue prior to disease onset).

A second novel intergenic locus with lower methylation levels in smokers was at 6p21.33 (chr 6: 307,020,080). This locus is in a gene desert which maps onto a DNase I hypersensitivity site and transcription factor binding site, associated with an H3K27 acetylated chromatin site, which is often associated with active regulatory elements. It is not associated with any gene or SNP. Further studies will be required to assess the functional nature of these two novel intergenic genomic loci.

Toxic components of cigarette smoke enter the bloodstream via the alveolar capillary system, after which they could directly affect the epigenetic profile of circulating WBCs. For the loci that correlated with the smoking status, we showed a strong correlation with time since quitting and duration of smoking, with methylation levels eventually returning to those of non-smoker levels. This is an observation that has been seen for methylation at the F2RL3 locus using the 27K methylation chip (20), and in the gene expression signature of smoking for some genes (21). Interestingly, the expression of some genes, including AHRR, does not return to previous levels, which indicates a long-term gene expression consequence of prior smoking history that may be locked in by DNA methylation changes (21). However, we have to consider the possibility that other confounding factors may influence this association. We and others have not adjusted for alcohol and body mass index, which may be smoking-associated or methylation-associated confounding factors. Furthermore, we have assessed methylation levels in the genomic DNA of all circulating WBCs. Smoking increases the circulating WBC count (22); although the effect of cigarette smoke on specific WBC types have not been assessed in large-scale populations, it may be that subsets with different methylation levels are clonally expanded due to the exposure, which might affect the overall methylation result. Using fractionation of blood cell types into T cells, B cells, monocytes, granulocytes and buffy coat (all leukocytes) compared with whole blood cell DNA from the same individuals we have shown no evidence that any of these blood cell types have significantly different methylation levels that would confound the association with smoking (Supplementary Material, Table S6). Lastly, genetic haplotype differences between individuals tagging allele-specific methylation may confound associations with the smoking status if such haplotypes are also associated with smoking (23). We have performed a preliminary analysis of 25 individuals for which we have both GWAS data and EWAS 450K methylation data (unpublished data). We found evidence for potential allele-specific methylation at the F2RL3 locus (general logistic regression $P = 0.01$), but not for the other smoking associated loci (Supplementary Material, Table S7). While the AHRR CG>CA SNP (cg23576855, presented in Fig. 3) does indeed influence the methylation of this site and is in linkage disequilibrium with the haplotype tag SNP, it does not influence allele-specific methylation of the haplotype, given that the two other nearby CpG sites (79 and 26 061 bp apart from the first site) show no evidence of allele-specific methylation ($P = 0.798$ and 0.916, respectively). Therefore, for the majority of loci it is unlikely that the methylation association with smoking reported in this study is due to genetic polymorphism.

Taken together, these studies report a strong link between tobacco use and a direct biological result on the epigenome, which is detectable in blood and lung tissue, and may impact on the cancer risk associated with smoking. The level of methylation was directly associated with the smoking intensity and duration; however, the biological consequences of these epigenetic alterations will require further investigation, particularly at these novel intergenic loci. The results of our study and others regarding a key gene in the AHR pathway may have relevance beyond tobacco smoke exposure, given the key role played by this pathway in the metabolism of many environmental carcinogens.

**MATERIALS AND METHODS**

**Subject recruitment**

Study participants were drawn from the Italian component of the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) cohort, a large general population cohort consisting of $\sim 520,000$ individuals with standardized lifestyle and personal history questionnaires, anthropometric data and blood samples collected for DNA extraction (24, 25). In this study, we used the Torino sub-cohort (EPIC-Turin) which consists of $\sim 10,000$ subjects. For the microarray component of this study, we included 92 incident female breast cancer cases and 92 matched controls and 95 incident colon cancer cases and 95 matched controls including 68 male and 27 female pairs. Controls were individually matched on age ($\pm$ 5 years), sex, seasonality of blood collection and duration of follow-up. Blood samples from cancer cases were taken 55 months before diagnosis on average (range 24–108 months) for breast cancer cases and 84 months before diagnosis (range, 0.2–173 months) for colon cancer cases (Supplementary Material, Table S5). Blood cell-type fractionation was performed on six healthy volunteers using magnetic bead separation (EasySep, StemCell Technologies, France).

**Microarray protocol**

For the microarray, DNA samples were extracted using the QIAasymply DNA Midi kit (Qiagen, Crawley, UK). Bisulphite conversion of 500 ng of each sample was performed using the EZ-96 DNA Methylation-Gold™ kit (Zymo Research, Orange, CA, USA). Bisulphite-converted DNA was used for hybridization on the Infinium HumanMethylation 450 BeadChip, following the Illumina Infinium HD methylation protocol. The methylation score for each CpG was represented as a $\beta$-value according to the fluorescent intensity ratio...
representing any value between 0 (unmethylated) and 1 (completely methylated). Raw microarray data and processed normalized data will be available from Gene Expression Omnibus (GEO) (accession TBA).

Validation of array-based methylation results by pyrosequencing

For the validation component of this study, 180 healthy control individuals were randomly sampled from females enrolled in the EPIC-Turin cohort. This group comprised 33 current smokers, 45 former smokers and 102 individuals who had never smoked. Genomic DNA (250 ng) from each subject was bisulphite converted as above. For pyrosequencing, specific primers were designed for six CpG loci using PyroMark software (Qiagen, Hilden, Germany), and PCR conditions were as described previously (26) (Supplementary Material, Table S6). Methylation values were calculated as an average of all high quality CpG sites, which were determined as ‘passed’ by the quality control thresholds within the Pyro Q-CpG software (Qiagen). Pyrosequencing assays for three loci (cg05951221, cg01940273 and cg05575921) could not be designed.

Human and murine expression analyses

Human lung samples (n = 27, 14 smokers, 13 non-smokers) were obtained from either lung transplants performed at The Royal Brompton or Harefield Hospital or purchased from IIAM (International Institute for the Advancement of Medicine, Edison, NJ, USA). In all cases, the tissue was consented for use in scientific research and ethics approval was obtained from the Royal Brompton & Harefield Trust. DNA was extracted and pyrosequencing was performed as described above. RNA was extracted from lung tissue (n = 5 smokers, n = 5 non-smokers), and quantitative RT-PCR (qRT-PCR) was performed on a Bio-Rad PCR machine with SyBR green (Sigma), for AHRR normalized against GAPDH, using standard protocols. Lung tissue was collected from 10 mice exposed to air (n = 5) or cigarette smoke (n = 5), as described previously (4). RNA was extracted and qRT-PCR was performed for Ahr, Ahn, Cyp1a1 and F2rl3 as described above, using a ribosomal gene, RpL7, as the internal reference standard.

Statistical analysis

For the statistical analysis, raw data were exported from GenomeStudio (Illumina) as background subtracted β-values with the corresponding detection P-values. Following quality control, the resulting datasets included 86 breast cancer cases with 87 matched controls (86 matched pairs) and 95 colon cancer cases and 95 matched controls. Overall, 484,804 probes were analysed in the breast cancer dataset and 485,152 in the colon cancer dataset. There were no significant genome-wide methylation differences between smokers, former and never smokers in raw β-values (Supplementary Material, Fig. S6); therefore, the data were normalized using quantile normalization. Multivariate linear regression was used to identify associations between methylation β-values as the outcome and the coded smoking status as the exposure (0, 1 and 2 for ‘Never’, ‘Former’ and ‘Current’ smokers, respectively), adjusting for age and batch. A secondary analysis was performed that also adjusted for the case–control status, which did not significantly alter the results (Supplementary Material, Table S2). P-values less than 1 × 10−7 were considered to be significant at the level of epigenome-wide significance. We found no evidence for a bias towards probes containing SNPs or type I or type II probes in probes associated with smoking. For association with smoking intensity, coded intensity categories were used as the exposure, Time to quitting and duration of smoking in former smokers were analysed as continuous variables. The attributable risk for the 2q37 locus was calculated as the difference in the rate of hypomethylation between an exposed population (cancer cases) and an unexposed population (healthy controls), stratified by smoking status. All analyses were performed in R, v2.13.1. Gene expression levels were compared between the control and smoke-exposed human or mouse lung tissue using Student’s t-tests with P < 0.05 considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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