Coordinated Changes in AHRR Methylation in Lymphoblasts and Pulmonary Macrophages from Smokers

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Abstract

Smoking is associated with a wide variety of adverse health outcomes including cancer, chronic obstructive pulmonary disease, diabetes, depression and heart disease. Unfortunately, the molecular mechanisms through which these effects are conveyed are not clearly understood. To examine the potential role of epigenetic factors in these processes, we examined the relationship of smoking to genome wide methylation and gene expression using biomaterial from two independent samples, lymphoblast DNA and RNA (n=119) and lung alveolar macrophage DNA (n=19). We found that in both samples current smoking status was associated with significant changes in DNA methylation, in particular at the aryl hydrocarbon receptor repressor (AHRR), a known tumor suppressor. Both baseline DNA methylation and smoker associated DNA methylation signatures at AHRR were highly correlated (r=0.94 and 0.45, respectively). DNA methylation at the most differentially methylated AHRR CpG residue in both samples, cg0557592, was significantly associated with AHRR gene expression. Pathway analysis of lymphoblast data (genes with most significant methylation changes) demonstrated enrichment in protein kinase C pathways and in TGF beta signaling pathways. For alveolar macrophages, pathway analysis demonstrated alterations in inflammation-related processes. We conclude that smoking is associated with functionally significant genome wide changes in DNA methylation in both lymphoblasts and pulmonary macrophages and that further integrated investigations of these epigenetic effects of smoking on carcinogenesis and other related co-morbidities are indicated.

INTRODUCTION

Despite extensive preventative and treatment interventions, approximately 19% of American adults smoke on a daily basis (Centers for Disease Control 2011). This is a substantial problem because smoking is the leading preventable cause of premature morbidity and mortality. Smoking causes approximately 450,000 premature deaths annually through its effects on the incidence of cancer, heart disease and chronic obstructive pulmonary disease (Center for Disease Control 2005). National data indicate that while both prevalence of smoking and mortality from lung cancer have significantly decreased for men between 1975 and 2007, these rates did not decrease for any racial or ethnic group or for women (Davis and others 2010). In addition, projections suggest that because women who were born

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around 1960 have higher prevalence of smoking and morbidity than other cohorts, this gender disparity may increase (Kohler and others 2011).

Many of the effects of smoking on the lung are thought to result from the direct effects of cigarette smoke on pulmonary epithelium and alveolar macrophages. However, the exact mechanism(s) through which smoking increases the risk for disease in non-pulmonary tissues such as blood and brain are unclear. Recently, sets of convergent findings have suggested that a portion of that vulnerability may be driven by differential DNA methylation acquired by smoking (Breitling and others 2011; Chang and others 2004; Philibert and others 2010; Suga and others 2008).

Altered DNA methylation that results from genetic lesions present at conception has long been established as a cause of disorders affecting early development of disease in the soma and the CNS. With respect to non-CNS disease, altered imprinting that usually results from maternal monosomy at 15Q causes Prader-Willi syndrome (Gurrieri and Accadia 2009). With respect to the CNS disease, almost all cases of Rett Syndrome result from mutations in MECBP2 which exert their effects by altering DNA methylation (Chahrour and Zoghbi 2007). Guided by clues such as the observations that addition of folate, a methyl donor, to the diets of pregnant women, markedly decreases the frequency of neural tube defects, the field has embraced the concept that alterations in DNA methylation may be associated with acquired early onset developmental disorders as well (Tsankova and others 2007). However, whether environmentally acquired alterations could increase likelihood of disease in adults has been an open question. A number of single gene and genome wide studies provide evidence that altered DNA methylation is associated with smoking and may be a cause of smoking associated illness. In particular, using both genome wide and single gene approaches, we and others have demonstrated that altered DNA methylation is associated with smoking (Breitling and others 2011; Chang and others 2004; Launay and others 2009; Philibert and others 2010; Suga and others 2008). However, these studies have been hindered by low coverage of the total number of genes and CpG residues in the human genome and discrepancies as to the appropriateness of certain forms of biomaterials for studies of epigenetic phenomena.

In this communication, we report our results with respect to smoking status on genome wide methylation and focal gene expression using two independent sets of biomaterials: 1) lymphoblast DNA and RNA derived from 119 female subjects from the Iowa Adoption Studies (IAS) and 2) alveolar macrophage DNA from cells isolated from the lungs of 10 smokers and 9 non-smokers.

METHODS

Human Subjects

The first set of biomaterials was obtained from subjects participating in the Iowa Adoptions Studies (IAS) (Yates and others 1998). In brief, the IAS is a case and control adoption study of the role of genetic, environmental and gene-environment interactions in the etiology of common behavioral illness. The clinical material used in the current study is derived from interviews with the Semi-Structured Interview for the Assessment of the Genetics of Alcoholism, Version II (Bucholz and others 1994), during each of the last two waves of the IAS study (1999–2004 and 2005–2009). The biological material used in this study, lymphoblast cell lines, was derived by Epstein Barr virus (EBV) mediated transformation (Caputo and others 1991) of lymphocytes obtained from blood donated by 165 female subjects during the last wave of the study.

The second set of biomaterials for the current study was alveolar macrophages obtained by bronchoalveolar lavage. Subjects were recruited from the community via advertisements and word-of-mouth. In order to be included, case (smoking) subjects had to be actively smoking with at least 10 pack year history of smoking. To be included as a control, the subject had to deny ever smoking cigarettes. Subjects were excluded if they had any significant co-morbid conditions such as pregnancy, or if a baseline spirometry revealed the Forced Expiratory Volume in the first second (FEV1) was less than 60% of predicted. All of these procedures and protocols were approved by the University of Iowa Institutional Review Board.

Bronchoalveolar Lavage

To obtain human alveolar macrophages a bronchoalveolar lavage was performed. After informed consent was obtained, subjects underwent standard flexible bronchoscopy. After the application of local anesthesia, bronchoalveolar lavage was performed by instilling 20 ml of normal saline into a tertiary bronchus up to five times in three different lung segments. The first collection out of five was discarded for possible contamination from upper airway secretions or by lidocaine, which is used to locally anesthetize the subject during the procedure. The remaining lavage was transported to the laboratory where fluid was filtered through sterile gauze and centrifuged at 200 x g for 5 min to pellet cellular material. The resulting pellet was suspended in phosphate buffered saline and centrifuged at 16,000 x g for one minute. The macrophages were suspended in medium, labeled with Wright stain and microscopically examined to ensure that greater than 95% of the cells were macrophages (Monick and others 2008; Monick and others 2006; Monick and others 2010).

DNA and RNA Isolation

The lymphoblast DNA and RNA used in this study was prepared from growth-entrained cell lines according to our standard procedures (Philibert and others 2008). In brief, on the day before DNA preparation, one-half of the cell media for each culture flask was exchanged. Twenty four hours later, DNA was prepared from the cell lines using cold protein precipitation. Simultaneously, RNA was purified from independent aliquots of the same culture using RNA Midi kits (Invitrogen, USA) according the instructions of the manufacturer. After quantification and purity assessment using a Nanodrop (Thermo Scientific, USA) spectrophotometer, DNA was stored at −20° C and RNA was stored at −80° C until use.

DNA and RNA were isolated from alveolar macrophages using the Qiagen DNAeasy™ kit (Qiagen, Valencia, CA) and MirVana (Applied Biosystems, Austin, TX) reagents according to manufacturer's instructions. Quality assessment was by Nanodrop and Experion (Bio-Rad Experion Automated Electrophoresis Station). After preparation, DNA was stored at −20° C and RNA was stored at −80° C until use.

DNA Methylation

Genome wide DNA methylation of the DNA was assessed using the Illumina HumanMethylation450 BeadChip under contract by the University of Minnesota Genome Center using the protocol specified by the manufacturer and the contractor. The resulting microarray data were inspected for complete bisulfite conversion of the DNA, and average beta values (i.e. average methylation) for each CpG residue were determined using the GenomeStudio V2009.2; Methylation module Version 1.5.5., version 3.2 (Illumina, San Diego). The resulting beta values were exported into Microsoft Excel and JMP (SAS Institute, USA) for data analysis. The HumanMethylation450 BeadChip contains 485,577 probes that recognize at least 20216 unique features (i.e. potential transcripts). With respect to this sample, >99.76 % of the 485,577 probes yielded statistically reliable data.

Data Analysis

After logarithmic conversion, data were inspected for outliers or confounding by plate or chip variables, and then the initial data analyses were conducted using genome wide t-tests. Subsequently, beta values for each of the probes were aligned according to their physical location and the data reanalyzed using paired t-tests over a 11-probe sliding window in order to more adroitly capture methylation signatures over larger regions (Dindot and others 2009; Farthing and others 2008). All genome wide comparisons were corrected for multiple comparisons using the method of Benjamini and Hochberg(Benjamini and Hochberg 1995). For select loci, data were analyzed with respect to alcohol use status using ANOVA (Fleiss 1981).

Pathway analysis of differentially methylated genes was conducted using GoMiner™ using default settings (0.05 settings for reports and all gene ontology as the root category setting) using the gene set specified in the text as the "changed" gene set (Zeeberg and others 2003). All values reported include nominal and FDR (false discovery rate) corrected values.

Specific qRT-PCR Analysis of AHRR

The relative expression of the aryl hydrocarbon receptor repressor (AHRR) was determined using primer probe sets from ABI, a Fluidigm BioMark™ System and proprietary BioMark Real-Time Analysis software according to manufacturer's guidelines. Briefly, first, RNA was converted to cDNA using an ABI cDNA archiving kit according to manufacturer's suggestions. Then after a brief pre-amplification step, each cDNA sample was amplified in quadruplicate with using primer probes for AHRR (Hs01005075) and five housekeeping genes (CALR, RPL7A, PRS19, RPS20 and UBC) obtained from Applied Biosystems (Foster City, USA). The Ct counts exported to the database, normalized using the geometric mean of five housekeeping genes, and then converted to Z scores for statistical analysis.

RESULTS

Iowa Adoption Study Cohort

The demographic and clinical characteristics of the 165 female subjects whose genome wide methylation status was assessed are shown in Table I. Overall, the subjects were largely white and tended to be in their mid-to-late 40s. Consistent with enrichment of the sample for the diathesis of substance use, the majority of the subjects in the study reported daily smoking at some period of their lives (85 of 165). However, many of these individuals (n=46) have quit smoking or were not smoking every day at the time of phlebotomy leaving only 39 subjects reporting daily smoking (i.e. seven days per week every week) at the time of phlebotomy. Because our prior studies have indicated that they methylation signature of those subjects who had recently quit smoking is highly variable, those 46 individuals were excluded from further study (Philibert and others 2010). The number of cigarettes smoked daily by the 39 subjects who smoked daily varied from 4 to 40 with the average number of cigarettes consumed daily being 19 cigarettes or about a pack per day for greater than 20 years. Cigarette smoking tended to be the only form of nicotine use currently being manifested by these 39 subjects with none of the subjects reporting the concomitant use of cigars, chew or other forms of nicotine usage in 2 weeks prior to assessment. There were no significant differences between the three groups (current smokers, never smokers, non-daily smokers/quitters) with respect to alcohol use in the past six months or age.

We contrasted the methylation values for the 39 smokers (average beta value 0.443) with the values for the 80 non-smokers (average beta value 0.446) using single point genome wide t-tests. The results of those analyses are shown in Table II. As the table indicates, only one probe, cg14817490, which maps to intron 3 of the of the aryl hydrocarbon receptor repressor

(AHRR) survived genome wide Benjamini-Hochberg correction for multiple comparisons. However, it is interesting to note that 3 other probes from AHRR, cg05575921, cg14454127, and cg03991871, were ranked among the top 13 probes and that none of them were from the rather small promoter associated CpG island. Instead, all 4 of the top AHRR probes target the gene body which contains three (>100 CpG residues) large CpG island according the UCSC genome browser. Finally, we note that cg03636183, a probe that was reported by Breitling and colleagues to be significantly associated with smoking status in lymphocyte DNA (Breitling and others 2011), was also nominally associated (p<0.003; rank 802nd of 485577 probes; smoker average 0.67; non-smoker average 0.74) with smoking status in the current study (Breitling and others 2011).

One possible concern is that some of the differential methylation signature could be secondary to alcohol use. Therefore, even though there were no significant differences between the rate of drinking for smoker and non-smoker groups, we analyzed the data for alcohol-related changes. The relationship of methylation to alcohol intake over the past 6 months to the methylation at loci controlling for alcohol use status was examined. Only two of the top 30 probes, cg07812589 and cg17231418, were even nominally related to amount of alcohol intake in the past 6 months, both at a p-value of $0.04< x<0.05$. Hence, there does not appear to be any effect of alcohol intake on the methylation status at the most differentially methylated loci (data available upon request).

Next, as part of our analyses, we conducted a sliding window analysis using an 11-probe window and the same groups of case and control subjects. Table III describes the result of those analyses. The addition of the methylation data immediately flanking each probe increased the overall significance of the findings with 36 comparisons surviving genome wide correction. Not surprisingly, many of the top thirty probes from the analysis tended to lie immediately adjacent to one another. Interestingly, despite the strength of four AHRR probes in the single probe analyses, the gene region containing these probes, which is interrogated by 149 separate markers, was not included in this list of top regions. Inspection of this locus shows that differential methylation was largely confined to the 2 or 3 probe windows surrounding each of these residues with each of these areas being several thousand base pairs apart (Supplemental Table I)..

Using GoMiner™, we conducted gene pathway analyses using the information from the 273 probes that were nominally differentially methylated at the p<0.001 level. Table IV shows the top 30 most differentially methylated pathways. Overall, only one pathway, protein kinase C (PKC) activity, survived false discovery rate (FDR) correction at the p<0.05 level. However, a recurrent theme of differential methylation in gene pathways affecting ion transport was found in many of the other less significant top thirty pathways.

Human Alveolar Macrophage Data

Because some may have concerns about the reliability of lymphoblast ability to model the changes found in their cognate lymphocytes and other primary cell types, we repeated these same case and control analyses using DNA from pulmonary alveolar macrophages again using a case and control paradigm. The case macrophages were isolated from the lungs of 10 smokers with at least a 10 year history of ≥ 1ppd smoking (6 male and 3 female) while the control macrophage biomaterial set was isolated from 9 non-smokers (6 male and 4 female). Although these two groups were roughly matched for ethnicity (smokers: 8 White, 2 African Americans; non-smokers: 9 White), the control group was significantly younger than the smoking group (smokers 31 ± 3 yrs, non-smokers 40 ± 4 yrs, p<0.01).

The results of the genome wide single probe contrasts are illustrated in Table V. Overall, the effects of smoking were much more profound with 1381 probes surviving correction for

genome wide comparison at a p<0.05 level. Of considerable interest given recent data suggesting a prominent role for AHRR in carcinogenesis, 8 probes from AHRR, including the 3rd ranked probe, cg25648203, were significantly associated after correction for genome wide comparisons. But of the top 4 AHRR probes from the lymphoblast analyses, only cg05575921 was significantly associated after Bonferroni correction.

We next repeated the sliding window analyses for the macrophage data using the same method delineated above. Once again, the results (see Table VI) were more robust than those for the lymphoblast data with 40 eleven probe regions being significantly associated after correction for multiple comparisons. Although many highly interesting genes were once again implicated in this analysis, AHRR was once again notable with the 28th ranked 11 probe region being found in the body of the AHRR.

As a last part of our set of analyses with respect to the macrophage methylation data, we repeated the GoMiner pathway analyses using the list of 1381 probes which were significantly associated in the above analyses as our changed gene set. Table VII shows those results of those analyses. In brief, pathways involved with wound healing, inflammation and G-protein/ras signaling were particularly prominent.

Comparison of Lymphoblast and Macrophage Data

In both the macrophage and lymphoblast analyses, probes from AHRR were repeatedly associated with smoking status. Therefore, we compared the methylation signatures from these two biomaterials with respect to smoking status. Supplemental Table I details the average methylation and single point analyses for each of the 146 probes for the gene for each biomaterial. In brief, 14 probes in the lymphoblast analyses and 40 of the probes in the macrophage analyses were associated with smoking status at a p<0.05 with 8 of the 14 probes in the lymphoblast analyses also being nominally significantly associated with smoking status in the macrophages with the direction of methylation being consistent at each probe (greater methylation in smokers). The overall methylation signature between the control lymphoblasts and macrophages at AHRR was highly correlated (r=0.95). Figure 1 illustrates the relationship between the differential methylation at each of the 146 residues listed in Supplementary Table I for the lymphoblast and macrophage DNA samples. As the figure shows, the differential methylation signature was also highly correlated across the gene with over 20% of the differential methylation signature that was associated with smoking status being shared between the two DNA sources (r=0.45; p<0.001).

An advantage of lymphoblasts is the ability to easily create high-quality RNA for gene expression studies. Therefore, to determine whether this differential methylation had functional consequences on lymphoblast gene expression, we then analyzed the relationship between AHRR gene expression and methylation status at cg05575921, the AHRR probe with the most consistent associations in the two analyses, using RNA prepared from the case and control samples. Interestingly, increasing methylation at this probe was associated with decreasing lymphoblast AHRR gene expression (p<0.03, n=108) which suggests that the CpG residues in this region may have a functional in vivo role in regulating gene expression at this locus.

DISCUSSION

In summary, we report that cigarette smoking is associated with significant changes in genome wide methylation, and in particular, AHRR methylation, in DNA derived from pulmonary alveolar macrophages and lymphoblasts. Strengths of this manuscript include confirmation of the findings from lymphoblast DNA, which are immortalized lymphocytes, with data from primary tissue from the lungs of smokers and the presentation of evidence

that these changes at AHRR may be functional. Possible limitations include the relative poor matching of the subjects who contributed lymphoblast and pulmonary macrophage DNA, occasional mis-annotations in the probe descriptor files, possible unaccounted effects of polymorphisms in the regions containing him the probes, and the fact that we did not verify the results with bisulfite sequencing.

The most significant and consistent finding in the current study is with respect to AHRR locus. AHHR is a feedback inhibition modulator of the aryl hydrocarbon receptor (AHR) that exerts its effects by competing with AHR for binding with its cognate nuclear receptor dimer partner (AHR nuclear translocator) or at xenobiotic response elements in AHR regulated genes (Haarmann-Stemmann and others 2007). This feedback modulation plays a pivotal role in AHR regulation and may be critical in moderating AHR role in oncogenesis and altered immune function (Opitz and others 2011). Our finding of smoking associated methylation at AHRR is highly plausible for several reasons. First and foremost, smoking is the leading preventable cause of cancer. Hence, this association may explain part of the connection. Second, the direction of the differential methylation was consistent among the 8 AHRR probes with nominal significance in both lymphoblast and macrophage comparisons with a high degree of shared smoking associated differential methylation (see supplemental table 1). Third, AHRR was the only gene locus that had significant localizations in both studies after correction for multiple comparisons. Fourth, previous studies have shown that smoking induces production of the AHR (Martey and others 2005; Meek and Finch 1999), a process which is thought to be critical for certain forms of smoking related forms of carcinogenesis (Andersson and others 2002; Gumus and others 2008; Shimizu and others 2000). Assuming that the decreased methylation at AHRR seen in smokers in the current study may result from a feedback mechanism associated with smoking induction of AHR transcription, the current findings are very consistent with previous findings and suggest potential avenues for addressing AHR mediated neoplastic transformation. Unfortunately, even given the promising gene expression findings, rigorous testing of this hypothesis may be difficult because review of the Ensembl and University of Santa Clara (UCSC) genome browser databases demonstrates the presence of three large CpG islands that are interspersed throughout the gene and at least 11 AHRR transcripts, each of which codes for a differently sized protein that may have unique competitive properties with respect to AHR. Hence, while the current findings are encouraging, a more definitive understanding of relationship between AHHR methylation and both AHRR gene expression and AHR function may require more complex and detailed examination of this region.

The pathway analyses of the macrophage data were illuminating and consistent with our understanding of the effects of smoking. The macrophage data was characterized by changes in inflammation, wound healing and Ras/G-protein signaling pathways. The repeated finding of altered methylation in Ras/G-protein signaling pathways seems logical since activation of these proteins are thought to be part of the oncogenic process for many types of cancers (Lewinski and Wojciechowska 2007; Tchevkina and others 2004). Similarly, the recurrent identification of wound healing and inflammatory pathways seems logical since smoking is the leading cause of Chronic Obstructive Pulmonary Disease (COPD), a syndrome in which the vast morbidity of the pathology is secondary to inflammatory moderated remodeling of the lung epithelium (Shapiro and Ingenito 2005). In contrast, the results of the lymphoblast analyses were less robust with only two pathways, related to peptidyl-threonine modification, surviving FDR correction. However, it is important to note that while both pathways are closely related with the basis of their significance in our analyses relying on the same five probes with the omission of one probe from either of these comparisons would result in nonsignificant findings.

The comparative weakness of the methylation findings in lymphoblasts as compared to macrophages highlight the importance of incorporating studies of primary tissues directly exposed to the substance in question. Overall, the smoking associated differential methylation was markedly more pronounced in the alveolar macrophage DNA than in the lymphoblast DNA. This is probably because circulating lymphocytes are less exposed to the direct effects of smoke than the macrophages resident in the lung. During cell replication, DNA methyltransferase 1 (DNMT1) stably copies cellular DNA methylation patterns (Suzuki and Bird 2008). However, it is possible that our conversion of these same lymphocytes into the transformed lymphoblast cell lines may further weaken the smoking induced signal. The latter possibility needs to be considered because although lymphoblast cell lines are excellent models of the lymphocytes from which they are derived, lymphoblast lines are vulnerable to clonal selection artifacts and there are well documented differences between lymphocyte and lymphoblast gene expression that occur as a function of EBV mediated transformation (Grafodatskaya and others 2009; Rollins and others 2010). Therefore, even though Vawter and colleagues have demonstrated that once transformed, gene expression profiles of lymphoblasts are relatively stable (Rollins and others 2010), the fact that the lymphoblasts by definition proliferate in non-smoking conditions, probably impact the data. To certain a certain extent this makes sense, if exposure to smoke induces an epigenetic change, the continued in vitro replication in the absence of smoking associated chemicals may mute the findings. This supports the importance of examining primary cells along with lymphoblasts.

It should also be recognized that most investigators, including Breitling and colleagues, use Ficoll separated mononuclear cell pellets rather than purified lymphocytes(Breitling and others 2011). Since these "lymphocyte pellets" contain a variety of cell types including B-lymphocytes, T-lymphocytes, monocytes and Natural Killer T-cell, it may well be that use of this heterogeneous cell mix may have obscured other potential findings which may explain why Breitling and colleagues only identified one differentially methylated probe in their study despite using a similar number of subjects.

Beyond the relative merits of lymphocyte and lymphoblast preparations, the current findings suggest that the lymphoblast lines paired with primary pulmonary macrophages will be useful in other investigations of the epigenetics of smoking because: 1) smoking has a broad effect on tissues throughout the body including the blood, and 2) integration of histone modification and gene expression status with DNA methylation status will require large numbers of cells. Some types of histone modification examinations necessitate relatively larger amounts of fresh cellular material. This suggests the utility of lymphoblasts in histone modification studies. A clear picture of lymphoblast gene expression and DNA methylation data relative to a primary smoking-relevant cell (alveolar macrophages) data will be needed for these potential future studies. In this respect, our convergent finding in lymphocytes and macrophages with respect to AHRR are reassuring.

One potential direction for future work is the determination of the specific AHRR transcripts that are differentially affected by differential methylation. The Taqman™ gene expression probe for AHRR used in this study (Hs01005075) recognizes the exon 3-4 exon boundary that is included in most splice variants. However, given the numerous splice variants produced by this gene, the epigenetic complexity of the gene (e.g. three large CpG islands not associated with the promoter), and its putative role in oncogenesis, future studies that examine specific splice variants altered by smoking is warranted.

The relationship of gene methylation to histone code modification should also be explored. In particular, the relationship of H3K4 and H3K27 methylation and H3K27 acetylation to AHRR gene expression should be examined because of the strong relationship of these

modifications to gene expression (Heintzman and others 2009; Kharchenko and others). Though DNA methylation is thought to have a weaker relationship to gene expression (Pai and others 2011; Wu and others 2010), if we can establish a stronger understanding of the histone-DNA modification relationship on a genome wide level, it well may be that we can use DNA methylation at loci such as AHRR as a proxy for histone status, and thereby gene expression status. Studies of DNA methylation are much cheaper and easier to conduct than histone modification studies. A better understanding of the relationship of peripheral blood methylation to methylation in other tissues, such as brain, may allow more informative studies of the role of DNA methylation and other forms of epigenetic changes in normal and disease related human development.

In summary, we report that cigarette smoking is associated with genome wide changes in lymphoblast and pulmonary macrophage DNA methylation, in particular at AHRR. We suggest replication and extension of the current findings and further investigations of the role of epigenetic changes in smoking altered gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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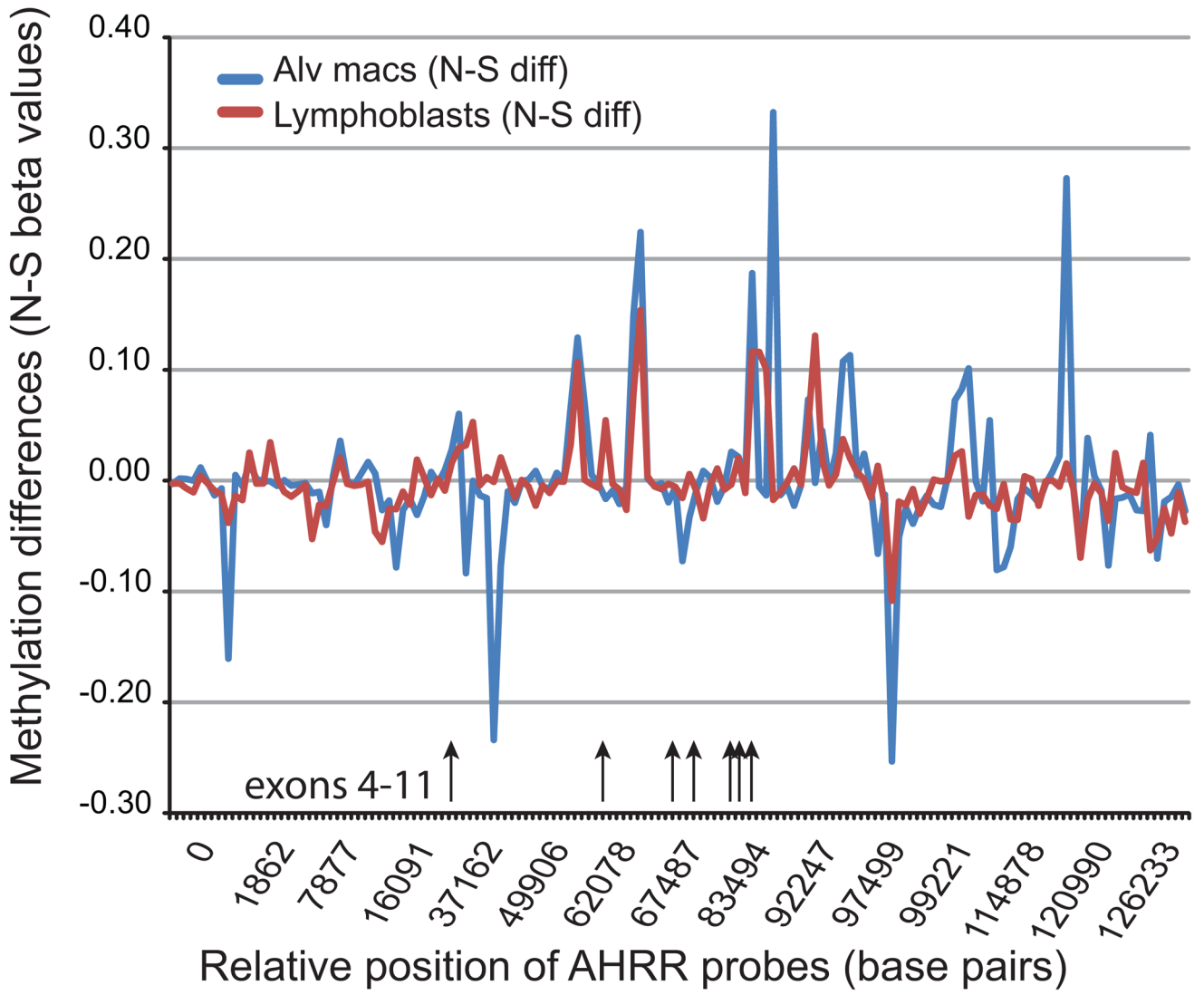
Figure 1.

Table I

Clinical Characteristics of the 165 Female Iowa Adoptions Studies Probands

	Non-Smoker	Quit or Quitting	Daily Smoker
<u>N</u>	80	46	39
<u>Age</u>	46 ± 8	47 ± 8	43 ± 6
<u>Ethnicity</u>			
White	80	44	39
Other	0	4	0
Alcohol in Past 6 months			
Yes	58	35	29
No	22	11	7
Daily Cigarette Usage			19 ± 9

Table II

The Top 30 Most Significantly Differentially Methylated Probes in Lymphoblast DNA.

Probe ID	GENE	Placement	Island Status	N-Smoker Avg	Smoker AVG	T-test	Corrected P-value
cg14817490	AHRR	Body		0.24	0.12	2.71E-08	0.02
cg05575921	AHRR	Body	N Shore	0.85	0.70	1.34E-06	0.29
cg07313705		S Shelf		0.07	0.10	1.78E-06	0.29
cg14454127	AHRR	Body		0.44	0.31	2.72E-06	0.34
cg02486161	NOD2	3'UTR		0.70	0.59	2.53E-05	0.99
cg14983684	RAD51L1	Body		0.75	0.71	2.58E-05	0.99
cg23939642	SLC38A10	Body		0.50	0.33	2.66E-05	0.99
cg25325005	PLEC1	Body	N Shelf	0.63	0.41	2.96E-05	0.99
cg23335946	C1orf251[st]	Exon	Island	0.08	0.09	3.14E-05	0.99
cg20776920	UNC5D	TSS1500	N Shore	0.87	0.83	3.21E-05	0.99
cg26812418	CPE	TSS200	Island	0.05	0.07	4.09E-05	0.99
cg07812589				0.26	0.20	4.59E-05	0.99
cg03991871	AHRR	Body	N Shore	0.78	0.67	4.97E-05	0.99
cg27545205		Island		0.02	0.02	5.26E-05	0.99
cg10951975	TRPM4	Body	Island	0.35	0.22	5.49E-05	0.99
cg20370184	SLC44A4	Body		0.27	0.12	5.64E-05	0.99
cg07999887	CPNE3	5'UTR	Island	0.02	0.02	5.92E-05	0.99
cg08644463	GNAI3	Body		0.87	0.83	6.83E-05	0.99
cg04366249	SGCE	1stExon	Island	0.05	0.07	7.34E-05	0.99
cg12741529	C3orf17	Body		0.87	0.85	7.75E-05	0.99
cg08940570	LOXL3	5'UTR	N Shore	0.80	0.66	9.09E-05	0.99
cg23754924	RGMA	Body	Island	0.10	0.13	9.56E-05	0.99
cg24547565	RUSC1	TSS1500	N Shore	0.51	0.62	9.85E-05	0.99
cg17093877	MGC16275	Body	N Shelf	0.57	0.43	0.00010	0.99
cg21545248	HMGXB3	Body		0.77	0.71	0.00011	0.99
cg22012583	LASS2	TSS1500	Island	0.37	0.25	0.00011	0.99
cg17231418	ESX1	Body	Island	0.26	0.39	0.00011	0.99
cg12668122	TMEM108	Body		0.40	0.31	0.00012	0.99
cg19776793	SLC38A10	Body		0.43	0.25	0.00013	0.99

Probe ID	GENE	Placement	Island Status	N-Smoker Avg	Smoker AVG	T-test	Corrected P-value
cg02724404	LYSMD4	TSS1500	S Shore	0.88	0.84	0.00013	0.99

All average methylation values are non-log transformed beta-values. Island status refers to the position of the probe relative to the island. Classes include: 1) Island, 2) N (north) shore, 3) S (south) shore, 4) N (north shelf), 5) S (south) shelf and 6) blank denoting that the probe does not map to an island.

Table III

The Top 30 Most Significantly Differentially Methylated Regions in Lymphoblast DNA.

Probe ID	GENE	Placement	Island Status	Average Methylation		P value[*]	Corrected P-value
				N-Smoker	Smoker		
cg13581859	HLA-DPB1	Body	Island	0.66	0.79	2.31E-09	0.002
cg25511667	HLA-DPB1	Body	Island	0.69	0.85	7.34E-09	0.002
cg14801692	HLA-DPB1	Body	Island	0.62	0.70	1.40E-08	0.003
cg03636880	HLA-DPB1	Body	Island	0.64	0.77	1.81E-08	0.003
cg01132696	HLA-DPB1	Body	Island	0.64	0.81	2.30E-08	0.003
cg10850215	HLA-DPB1	Body	Island	0.64	0.76	3.07E-08	0.003
cg02692313	HLA-DPB1	Body	Island	0.66	0.83	4.14E-08	0.003
cg03229061	HLA-DPB1	Body	Island	0.62	0.71	4.53E-08	0.003
cg17588455	HLA-DPB1	Body	Island	0.62	0.73	5.55E-08	0.003
cg19990651	HLA-DPB1	Body	Island	0.65	0.83	6.80E-08	0.004
cg14870156	HLA-DPB1	Body	Island	0.66	0.79	7.47E-08	0.004
cg06437840	HLA-DPB1	Body	Island	0.52	0.69	8.16E-08	0.004
cg26645432	HLA-DPB1	Body	Island	0.71	0.86	1.00E-07	0.004
cg20223237	HLA-DPB1	Body	Island	0.73	0.88	1.24E-07	0.005
cg25796439	ISM1	TSS1500	Island	0.08	0.08	1.26E-07	0.006
cg12893780	HLA-DPB1	Body	Island	0.67	0.82	1.84E-07	0.006
cg19759481	HOXA5	TSS200	Island	0.63	0.54	1.99E-07	0.007
cg04863892	HOXA5	TSS200	Island	0.68	0.60	2.53E-07	0.008
cg01992382	TNXB	Body	Island	0.42	0.47	2.74E-07	0.008
cg01370449	HOXA5	TSS200	Island	0.69	0.63	3.11E-07	0.010
cg12746059	PCDH10	TSS200	Island	0.08	0.09	3.95E-07	0.02
cg13349035	HLA-DPB1	Body	N Shore	0.68	0.80	4.72E-07	0.02
cg09549073	HOXA5	5'UTR	Island	0.68	0.60	6.91E-07	0.02
cg02916332	HOXA5	TSS1500	Island	0.64	0.58	7.89E-07	0.02
cg12128839	HOXA5	TSS200	Island	0.56	0.47	8.21E-07	0.02
cg17569124	HOXA5	TSS1500	Island	0.57	0.48	8.90E-07	0.02
cg06831576	CDH8	TSS200	Island	0.11	0.15	1.00E-06	0.02

| Probe ID | GENE | Placement | Island Status | Average Methylation | | P value[*] | Corrected P-value |
				N-Smoker	Smoker		
cg04525757	FOXG1	TSS1500	N Shore	0.14	0.15	1.25E-06	0.03
cg26242583	LUZP2	TSS200	Island	0.11	0.13	1.35E-06	0.03
cg19714132	FOXG1	TSS1500	N Shore	0.19	0.21	1.58E-06	0.03

[*] Nominal P-value before Benjamini-Hochberg correction. Corrected value is per Benjamini-Hochberg method.

Table IV

The Top 30 Most Differentially Regulated Pathways in Lymphoblast DNA

GO Category	Category Name	Genes		Log^{10} P-Value	FDR
		Total	Changed		
GO:0018107	peptidyl-threonine phosphorylation	27	5	−5.03	0.01
GO:0018210	peptidyl-threonine modification	30	5	−4.80	0.01
GO:0060914	heart formation	9	3	−4.00	0.09
GO:0009653	anatomical structure morphogenesis	1490	31	−3.53	0.15
GO:0045121	membrane raft	160	8	−3.45	0.14
GO:0007548	sex differentiation	181	8	−3.10	0.24
GO:0005024	TGF beta receptor activity	18	3	−3.05	0.20
GO:0007530	sex determination	18	3	−3.05	0.20
GO:0003007	heart morphogenesis	104	6	−3.03	0.18
GO:0004675	transmembrane receptor protein serine threonine kinase activity	19	3	−2.97	0.19
GO:0003197	endocardial cushion development	5	2	−2.94	0.22
GO:0005026	TGF beta receptor activity type II	5	2	−2.94	0.22
GO:0060021	palate development	46	4	−2.82	0.26
GO:0051015	actin filament binding	48	4	−2.75	0.33
GO:0030501	pos. reg. of bone mineralization	23	3	−2.73	0.33
GO:0070169	pos. reg. of biomineral tissue dev.	24	3	−2.67	0.33
GO:0003128	heart field specification	7	2	−2.63	0.31
GO:0003129	heart induction	7	2	−2.63	0.31
GO:0051864	histone demethylase activity	7	2	−2.63	0.31
GO:0061311	cell surface receptor linked signaling pathway involved in heart dev.	7	2	−2.63	0.31
GO:0060389	SMAD protein phosphorylation	25	3	−2.62	0.30
GO:0001649	osteoblast differentiation	86	5	−2.62	0.29
GO:0005901	caveola	53	4	−2.59	0.29
GO:0031095	platelet tubular network membrane	8	2	−2.51	0.32
GO:0035173	histone kinase activity	8	2	−2.51	0.32
GO:0046541	saliva secretion	8	2	−2.51	0.32
GO:0045669	pos. reg. of osteoblast differentiation	28	3	−2.48	0.33
GO:0070838	divalent metal ion transport	229	8	−2.45	0.32

		Genes			
GO Category	Category Name	Total	Changed	Log^10 P-Value	FDR
GO:0045778	pos. reg. of ossification	29	3	−2.43	0.33
GO:0030154	cell differentiation	2041	35	−2.43	0.32

dev.= development, pos. reg. = positive regulation, FDR=false discovery rate.

Table V

The Top 30 Most Significantly Differentially Methylated Probes in Alveolar Macrophage DNA.

Probe ID	GENE	Placement	Island Status	N-Smoker Avg	Smoker AVG	T-test	Corrected P-value
cg06961313	MR1	TSS1500		0.80	0.57	1.06 E-10	5.16201E-05
cg00738897			S_Shore	0.71	0.55	1.90 E-09	0.0003
cg25648203	AHRR	Body		0.38	0.72	1.97 E-09	0.0003
cg00506299	RFTN1 Body			0.23	0.46	2.67 E-09	0.0003
cg27229484	ZC3H12A	Body		0.26	0.53	3.34 E-09	0.0003
cg05951221		Island		0.28	0.42	5.85 E-09	0.0005
cg01432692				0.20	0.37	7.69 E-09	0.0005
cg09374353	EHD1	3'UTR	N_Shore	0.12	0.39	1.05 E-08	0.0006
cg14310198	RAPGEF1	Body		0.48	0.70	1.90 E-08	0.0010
cg21566642		Island		0.33	0.56	2.12 E-08	0.0010
cg17576603	DAB2	5'UTR		0.39	0.62	3.36 E-08	0.0013
cg17574812	ABHD6	Body		0.26	0.49	3.55 E-08	0.0013
cg06634140				0.30	0.54	3.73 E-08	0.0013
cg11254522	FGR	Body		0.35	0.50	3.99 E-08	0.0013
cg07457727			N_Shelf	0.22	0.60	4.02 E-08	0.0013
cg13458803	CD80	5'UTR		0.36	0.16	4.86 E-08	0.0014
cg01668352	SRGAP1	Body		0.32	0.62	4.97 E-08	0.0014
cg04402828	KIAA1026	Body		0.47	0.35	6.69 E-08	0.0018
cg07650681	LOC100132354	Body		0.66	0.40	7.19 E-08	0.0018
cg13610455	LOC388796	Body		0.29	0.44	7.37 E-08	0.0018
cg09127592	TRIM35	Body	N_Shelf	0.33	0.73	8.72 E-08	0.0019
cg14223856				0.43	0.81	9.60 E-08	0.0019
cg09006487	RYBP	3'UTR		0.35	0.50	9.69 E-08	0.0019
cg02233197	TNFAIP8L3	Body	S_Shelf	0.29	0.72	9.85 E-08	0.0019
cg05317600				0.34	0.65	9.86 E-08	0.0019
cg25466245	SUSD4	Body		0.36	0.57	1.09 E-07	0.0020
cg21418854	C1orf113	TSS1500	N_Shore	0.42	0.58	1.13 E-07	0.0020
cg02341139			S_Shelf	0.34	0.60	1.17 E-07	0.0020
cg18030943	LAMP3	Body	N_Shelf	0.23	0.38	1.20 E-07	0.0020

Probe ID	GENE	Placement	Island Status	N-Smoker Avg	Smoker AVG	T-test	Corrected P-value
cg05337681	LIPC	Body		0.23	0.47	1.25 -07	0.0020

All average methylation values are non-log transformed beta-values. Island status refers to the position of the probe relative to the island. Classes include: 1) Island, 2) N (north) shore, 3) S (south) shore, 4) N (north shelf), 5) S (south) shelf and 6) blank denoting that the probe does not map to an island.

Table VI

The Top 30 Most Significantly Differentially Methylated Regions in Alveolar Macrophage DNA

Probe ID	GENE	Placement	Island Status	Average Methylation		P value[*]	Corrected P-value
				N-Smoker	Smoker		
cg07965566				0.60	0.30	2.40E-28	1.16E-22
cg14310198	RAPGEF1	Body		0.70	0.48	4.83E-26	1.17E-20
cg17574812	ABHD6	Body		0.49	0.26	9.83E-25	1.59E-19
cg01668352	SRGAP1	Body		0.62	0.32	1.76E-24	2.14E-19
cg17576603	DAB2	5'UTR		0.62	0.39	2.52E-23	2.45E-18
cg07457727				0.60	0.22	3.98E-21	3.22E-16
cg101169462				0.13	0.06	1.30E-15	9.05E-11
cg24790419	KIAA1683	TSS1500		0.62	0.39	1.54E-15	9.38E-11
cg04402828	KIAA1026;	Body		0.35	0.47	3.16E-15	1.70E-10
cg05951221				0.42	0.28	4.80E-12	2.33E-07
cg16039867	MKNK1	Body		0.58	0.78	2.50E-11	1.10E-06
cg06634140				0.54	0.30	9.81E-10	3.97E-05
cg20485084	FGR	Body		0.67	0.36	1.91E-09	7.16E-05
cg02341139				0.60	0.34	6.35E-09	0.0002
cg22019569	SMYD3	Body		0.60	0.75	1.38E-08	0.0004
cg14019523	ASB2	Body		0.37	0.24	2.11E-08	0.0006
cg13675814	CORO2A	5'UTR		0.67	0.82	6.95E-08	0.0018
cg04307274				0.57	0.31	6.96E-08	0.0018
cg15149645	NUPR1	TSS200		0.59	0.75	9.66E-08	0.0024
cg10192877	ABCG1	Body		0.61	0.74	1.20E-07	0.0028
cg21566642				0.56	0.33	1.24E-07	0.0028
cg20568305	GRAMD4	Body		0.68	0.52	2.95E-07	0.0065
cg01432692				0.37	0.20	3.35E-07	0.0070
cg24446429	MBP	Body		0.60	0.39	3.88E-07	0.0075
cg14414943	CHI3L2	Body		0.83	0.89	3.89E-07	0.0075
cg16659773				0.56	0.43	4.16E-07	0.0077
cg04135110	AHRR	Body		0.15	0.38	5.29E-07	0.0095

Probe ID	GENE	Placement	Island Status	Average Methylation		Smoker	P value [*]	Corrected P-value
				N-Smoker				
cg00738897				0.55		0.71	6.03E-07	0.0104
cg13458803	CD80	5'UTR		0.16		0.36	7.97E-07	0.0133
cg11691844	SYTL2	Body		0.27		0.44	8.63E-07	0.0139

[*] Nominal P-value before Benjamini-Hochberg correction. Corrected value is per Benjamini-Hochberg method.

Table VII

The Top 30 Most Differentially Regulated Gene Ontology Pathways in Macrophage DNA

GO Category	Category Name	Genes		Log^{10}	P-Value	FDR
		Total	Changed			
GO:0005737	cytoplasm	7845	429	−8.40	0	
GO:0007165	signal transduction	2324	159	−7.89	0	
GO:0005515	protein binding	6815	378	−7.62	0	
GO:0023052	signaling	3788	233	−7.56	0	
GO:0023033	signaling pathway	2813	183	−7.48	0	
GO:0007264	small GTPase mediated signal trans.	566	54	−6.90	0	
GO:0023060	signal transmission	2728	173	−6.29	0	
GO:0023046	signaling process	2730	173	−6.27	0	
GO:0009611	response to wounding	828	68	−6.06	0	
GO:0030234	enzyme regulator activity	880	71	−6.02	0	
GO:0030695	GTPase regulator activity	437	43	−5.96	0.001	
GO:0035466	reg. of sig. pathway	1158	87	−5.96	0.001	
GO:0051056	reg. of GTPase mediated sig. trans.	339	36	−5.85	0.001	
GO:0023034	intracellular sig. pathway	1708	117	−5.81	0.001	
GO:0060589	nucleoside-triphosphatase reg. activity	446	43	−5.73	0.001	
GO:0006928	cellular component movement	687	58	−5.61	0.001	
GO:0044444	cytoplasmic part	5629	311	−5.60	0.001	
GO:0007265	Ras protein signal transduction	335	35	−5.54	0.001	
GO:0016192	vesicle-mediated transport	743	61	−5.48	0.001	
GO:0010876	lipid localization	188	24	−5.44	0.001	
GO:0005085	guanyl-nucleotide exchange factor act.	152	21	−5.38	0.001	
GO:0035556	intracellular signal transduction	1454	101	−5.31	0.001	
GO:0006869	lipid transport	167	22	−5.26	0.001	
GO:0010885	reg. of cholesterol storage	12	6	−5.24	0.001	
GO:0016477	cell migration	564	49	−5.16	0.001	
GO:0042060	wound healing	470	43	−5.15	0.001	
GO:0007166	cell surface receptor linked sig. path	1745	116	−5.14	0.001	
GO:0005089	Rho guanyl-nucleotide exchange act.	68	13	−5.08	0.001	

		Genes				
GO Category	Category Name	Total	Changed	Log^{10}	P-Value	FDR
GO:0051179	localization	3540	207	−5.02	0.002	
GO:0001816	cytokine production	240	27	−4.99	0.002	

pos. reg. = positive reg., sig. =sig., trans. = transduction, FDR=false discovery rate.