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The DNA Methylation Signature of Smoking: An Archetype for the Identification of Biomarkers for Behavioral Illness

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Abstract

Smoking is perhaps the foremost public health challenge in the United States and in the world. In a series of rapidly emerging studies, we and others have demonstrated that cigarette smoking is associated with changes in the DNA methylation signature of peripheral blood cells. The changes associated with this type of substance use are both dose and time dependent. These changes in DNA methylation are also accompanied by changes in gene transcription and protein expression whose patterns are furthermore indicative of increased vulnerability to other forms of complex illness. In the past, our efforts to translate this knowledge into actionable information has been stymied by a lack of methods through which to systematically assess these changes. The rapid advance of DNA methylation assessment technologies changes that dynamic and presents the possibility that methylation-based clinical tools to aid the ascertainment of smoking status or effectiveness of treatment can be developed. In this chapter, we will review the latest advances in this field and discuss how these advances allow us insight as to methods through which to prevent smoking and shed insight into optimizing strategies through which to identify biomarkers for other behavioral illnesses which share similar contributions from environmental and gene-environmental interaction effects.

INTRODUCTION

Smoking is the most common preventable cause of morbidity and mortality in the United States and throughout the developed world. In the United States alone, smoking causes 440,000 premature deaths and \$100 billion of lost economic activity annually. (Centers for Disease & Prevention, 2008) Smoking, like most environment processes, does not exert these effects directly. Rather, smoking exerts its effects through increasing vulnerability to common complex medical disorders such as coronary artery disease (CAD), chronic obstructive pulmonary disease (COPD), Type 2 Diabetes (T2DM) and cancer. Hence, the true costs of smoking are reflected in the increased morbidity and mortality from these disorders. However, it is important to note that many of these increased smoking associated risks for CAD, COPD and cancer are potentially reversible upon cessation of smoking. (U.S. Department of Health and Human Services, 1990) This suggests the possibility that

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reversible cell characteristics also may result from smoking and that by understanding these modifications, we could gain both a better understanding of the effects of smoking and the cellular mechanisms underlying vulnerability to existing common complex medical disorders.

A Rationale for Continued Investigations

In the current healthcare environment, which rewards the development of interventions to prevent unwanted effects of the environment, the potential for development of new pharmaceuticals to stop smoking or prevent its consequences guides many existing studies of smoking. However, there is also an opportunity to examine basic processes in the context of smoking and perhaps shed light on etiological and recovery processes for a number of complex disorders. In particular, because many other environmental factors such as adverse childhood events may act through similar basic mechanisms to increase vulnerability to complex medical disorders, it is possible that the study of the mechanisms through which smoking alters vulnerability may provide insight into shared mechanisms through which smoking and these other environmental disorders interact with genetic variation to influence the likelihood of disease.

In order to better understand the spread of methylation changes, and so enhance potential for future pharmacological intervention to prevent health consequences, we will need repeated global assessments of cellular and somatic function. By necessity, these studies will need to be broad-based. It is unlikely that the cigarette smoke uniquely affects only one biological pathway. Cigarette smoke may contain over 4800 substances-each of which has its own unique effects.(Adam, McAughey, Mocker, McGrath, & Zimmermann, 2010) These effects on cellular substrates are extremely diverse. For example, many of these chemicals exert their effects by post-translationally modifying existing proteins or forming potentially mutation inducing adducts with DNA.(Van Schooten et al., 1997) Because many of these interactions are non-specific and tend to target specific amino acids or base pairs, rather than specific proteins or genes, the effects tend to be widespread across many potential pathways. But direct chemical modification of cellular substrates is not the only mechanism of disease. Many of the physiological changes associated with smoking may be secondary to inflammation associated with particulate matter rather than direct chemo-toxic effects. (Wannamethee et al., 2005)

No matter how wide-ranging, global assessments of cellular and somatic function also will have to be chronologically informed. Many of the effects of cigarette smoke are dose dependent and the pathological changes associated with exposure to that smoke tends to occur in a rather orchestrated fashion. For example, with respect to respiratory processes, the first changes evident are chronic coughs, which are then followed by recurrent bronchitis which then progresses in a subset of individuals to COPD.(Yawn & Kaplan, 2008) Hence, there may be a temporal order or threshold for the response of various biological systems to cigarette smoke.

However, there is a second set of reasons for understanding the modifiable processes associated with smoking; the development of better prevention and treatment measures. Currently, there are two principal mechanisms through which smoking is detected. The first

is self-report; the second is assessment of biomarker status. Each of these methods is potentially flawed.(Florescu et al., 2009) Self-report tends to work modestly well in adult epidemiological samples. Unfortunately, when done with younger samples, self-report tends to be unreliable. For example, in a survey of representative sample of 2107 American adolescents between 12 and 17 years of age, in which a relatively high cut off point (11.4 ng/ml) for serum cotinine employed as the “gold standard”, 3% of the self-reported nonsmokers were positive for tobacco use.(Caraballo, Giovino, & Pechacek, 2004) Since the overall rate of self-reported smoking in the sample was only 12%, indicating an overall sensitivity of only 80%. In a similarly structured larger British study that examined 6984 adolescents between the ages of 12–15 years, similar levels of sensitivity and relatively modest sensitivity being observed depending on the serum cotinine cut-off point employed. (Jarvis, Fidler, Mindell, Feyerabend, & West, 2008) In pregnant women, the results with self-report of smoking are even more troubling. Using very conservative cutoffs for this biomarker, four groups have examined the misclassification rates (high cotinine level with negative self-report) in pregnant women and found misclassification rates of 73%, 17%, 4% and 26%(Boyd, Windsor, Perkins, & Lowe, 1998; Britton, Brinthaup, Stehle, & James, 2004; Owen & McNeill, 2001; Webb, Boyd, Messina, & Windsor, 2003). In a recent review of these studies, Boyd and colleagues reviewed these four studies and concluded that if normal cutoffs had been employed, the level of misclassification may have been considerably higher.(Gorber, Schofield-Hurwitz, Hardt, Levasseur, & Tremblay, 2009)

Disappointingly, the use of the two most commonly employed biomarkers does not fully address the shortcoming of self-report. The assessment of cotinine level is probably the most commonly determined biomarker. Cotinine is the metabolite of nicotine and has a serum half-life of approximately 15–19 hours(Benowitz, 1996). Cotinine can be assessed in a wide variety of samples including those of blood, urine and hair. However, cotinine determinations do have their shortcomings. First, because of the half-life of cotinine, it is not useful for the detection of light smokers or periodic smokers such as “chippers”.(Florescu et al., 2009) This insensitivity is particularly troubling because it precludes its use in the detection individuals in the earliest phase of smoking which are thought to be the most tractable for treatment. Second, because nicotine in nicotine replacement therapy (NRT) vehicles such as the nicotine patch or gum are used by the vast majority of subjects in nicotine cessation therapy, cotinine assessments are not useful for monitoring response to treatment in smoking cessation therapy.

A second biomarker for smoking is assessment of exhaled carbon monoxide (CO). Exhaled CO assessment is easy to conduct and there are several easy to use devices for assessing CO in the clinical setting. Unlike the assessment of cotinine, CO assessments of smoking status are not obfuscated by the use of NRT. However, because of the short half-life of CO in the blood (3–4 hours), these assessments are only sensitive to the use of cigarettes in the past 8–12 hours(Stevens & Munoz, 2004). Hence, neither exhaled CO nor serum cotinine assessment is sensitive to low frequency smoking and it is quite possible that those who wish to avoid detection can do so by simply abstaining from cigarette use for 24–48 hours.

In summary, although both cotinine and CO assessments can serve vital roles in the assessment of current smoking status, they have significant shortcomings. Therefore, a

second objective which could be obtained through a better understanding the molecular consequences of smoking is the development of a better biomarker for the prevention and treatment of smoking.

Searching for the Biological Underpinning of Smoking

Similar to the searches for other disorders, the quest for the biological determinants of smoking and smoking associated factors began with smaller, less well powered twin and family studies, progressing eventually to the large multi-cohort meta-analyses evident in the recent literature.(Li, 2006; Liu et al., 2010) Each of these studies has had a role in defining our understanding of the cellular and somatic mechanisms of illness. The earlier family and twin studies have been informative in a number of manners. First, they have demonstrated that in the current environment, approximately 40–50% of the total vulnerability to smoking results from heritable genetic factors.(Lesso et al., 2004; Li, 2006; Maes et al., 2006; True et al., 1999) Second, they have demonstrated that the strength of those factors is environmentally contextual. In simple terms and now obvious in retrospect, genetic factors lead to smoking only if exposure to cigarettes occurs. Third, they have shown that the strength of genetic factors is age dependent. Genetic factors exert their peak influence in late adolescence but are largely without effect with respect to those 35 years or older(Heath, Kirk, Meyer, & Martin, 1999). Though these earlier studies did not identify specific genetic variants, they have been extremely informative for construction of evidence based preventative public policy measures. As such, the cost of conducting the studies has more than amply been recovered.

The advent of genome wide association techniques and the availability of large cohorts of informative populations has continued to transform our understanding of the genetics of smoking and other complex behavioral illnesses. At the current time, meta-analyses of cohorts including over 40,000 subjects has demonstrated that the predisposition to smoking is conveyed by a large number of small effect variants such as that of those found in the nicotinic receptor cluster at 15q13.(Liu et al., 2010) Unfortunately, the polymorphisms at this cluster seem to be the only ones detectable at currently available sample sizes. Furthermore, while they confirm our understanding of the processes underlying addiction, they do not inform us of the mechanisms underlying smoking associated illness nor predict with any degree of certainty who is likely to be a smoker. In contrast, analyses of candidate genes for other disorders, such as COPD, has demonstrated that factors that directly predispose to those other illnesses in the absence of smoking, act synergistically with smoking to increase the likelihood of disease(Hancock et al., 2012). Still, though less illuminating than hoped, these studies have confirmed prior assessments that in many cases cigarette smoking may augment existing pathological processes to cause common complex medical illness and that by understanding the effects of smoking, we may derive greater insight into the pathophysiological processes underlying these secondary disorders.

If understanding the genetics of smoking itself does not generate insight into the biology of smoking associated illnesses, what approach might be more tractable? As noted above, one potential insight into causative mechanisms is given by the remission of risk for certain smoking associated disorders upon remission of smoking-in so many words, the effects on

cell behavior must be modifiable. The second potential mechanism can be gleaned from the behavior of cells such as alveolar macrophages in response to smoking. Alveolar macrophages are derived from peripheral blood monocytes and migrate into the lung in response to inflammation. Upon stimulation, these monocytes transform into M1 and finally M2 macrophages.(Shaykhiev et al., 2009) Type I macrophages are associated with inflammation while type II macrophages are associated with the tissue remodeling found in COPD. Each of these relatively stable transformations, or changes in cell fate, is associated with changes in morphology and gene expression.(Shaykhiev et al., 2009) Because these changes are very predictable in the response to smoking, it is unlikely that they are secondary to the random mutagenic effects of smoking related carcinogens. Rather, it is likely that the cell fate changes observed in such diverse tissues such as the lung and arterial intima result from changes in the epigenetic programming of cells.

An Epigenetic Approach to Smoking

Epigenetics is the study of changes of gene expression or cell fate that do not involve changes in primary DNA sequence. Although the importance of epigenetic processes has been appreciated for a considerable period of time, the wide spread availability of tools for the dissection of epigenetic factors has only been available for the past decade. Our understanding of the factors contributing the “epigenetic code” continues to evolve. Traditionally, this knowledge base has focused on the role of DNA methylation and histone modifications in regulating the symphony of gene transcription. DNA methylation is probably the best understood of these epigenetic mechanisms and is thought to have arisen as a method of protection against the replication of retroposons (e.g., line-1 elements) or endogenous retroviruses.(Suzuki & Bird, 2008) In mammals, methylation is mediated by one of three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B). These enzymes catalyze the transfer of a methyl group from a methyl donor, such as S-adenosylmethionine (SAM), to the carbon-5 position of a cytosine-guanosine (CpG) dinucleotide pair.

Methylation of CpG residues in CpG islands is particularly important. Overall, the frequency of CpG dinucleotide pairs in the human genome is only 4% of the expected arithmetic value. However, there are approximately 30–40,000 areas in the human genome, known as CpG islands, in which high frequencies of CpG residues are found.(Suzuki & Bird, 2008) Approximately 2/3 of these CpG islands tend to occur immediately adjacent to gene promoters.(Fan & Zhang, 2009) Increases in the amount of methylation in these promoter associated CpG islands usually (but not always) results in the down regulation of RNA transcription from downstream genes. The mechanism through which this downregulation of expression is accomplished is thought to occur largely through steric means. Increased methylation of a region is associated with the recruitment of histones, such as histone type 3 (H3K27), and incorporation into tightly wound nucleosomes that are largely inaccessible to RNA Pol II.(Park & Nakai, 2011) Since CpG methylation and the associated processes can also recruit factors associated with chromatin remodeling, the methylation of these promoter CpG residues can act synergistically with other regulatory mechanisms such as histone modifications to encourage further transcriptional down regulation of adjacent gene regions.

DNA methylation acts in an iterative fashion with other epigenetic factors to modulate gene expression. In general, the type of histone modifications that are associated with CpG methylation associated silencing are the tri-methylation of the lysine residue of histone type 3 (H3K27) and deacetylation of H3K27 (Park & Nakai, 2011). However, a full understanding of the role of 41 known post-translational modifications is beyond the scope of this review and it is important to note that our global understanding of epigenesis continues to evolve has taken recently taken another interesting twist with the discovery that not DNA but RNA undergoes epigenetic modification. (Meyer et al., 2012) Therefore, the reader is referred elsewhere for the most recent advances in our understanding of epigenetic factors such as RNA methylation, micro RNA (including the recent discovery of circular microRNA) and quaternary structure. (Smith & Meissner, 2013) However, since it is clear that: 1) epigenetic factors work in concert, 2) the majority of gene expression can be derived by the assessment of existing factors, and 3) tools for the systematic exploration of the effects of some epigenetic factors in humans do not yet exist; current exploratory efforts will have to continue to rely on assessment of DNA methylation with an eye to future scalable assessments of other factors.

Assessing DNA Methylation Status

The methods through which methylation is assessed have steadily improved over the past decade. The development of bisulfite sequencing techniques in the 1990's was a major step forward in the characterization of bisulfite status. (Thomassin, Oakeley, & Grange, 1999) However, secondary to technical difficulties with bisulfite sequencing such as incomplete conversion of unmethylated cytosine residues and the instability of bisulfite converted DNA, many investigators chose to use digestion of DNA by methyl sensitive restriction endonucleases as the principal means for characterizing complex genomes. However, over the past several years, a number of platforms for the large-scale interrogation of methylation status have emerged including the Illumina 450K Human MethylationBeadChip and reduced representation bisulfite sequencing (RRBS) (for review, see Bock et al. 2010 (Bock et al., 2010)). These and other single locus techniques have led to a quantum leap in our understanding of the role and methylation in both cellular function and human complex disease. These illnesses include traditional complex medical disorders such as cancer and diabetes as well as complex neuropsychiatric disorders.

These advances have built on a steadily increasing body of literature implicating altered epigenetic status in the development of childhood onset neuropsychiatric disorders. One of the most seminal arguments was advanced in 1995, when it was established that established that uniparental disomy at 15q11-q13 that causes Angelman's and Prader-Willi Syndrome exerts its effects through altering epigenetic status. (Gurrieri & Accadia, 2009) Following the discovery in 1999 that mutations in methyl-CPG binding protein 2 (MECP2), which is known to bind methylated cytosines and recruit silencing complexes, was the cause of Rett Syndrome (Weaving, Ellaway, Gécz, & Christodoulou, 2005), a large number of investigators began to postulate that other neuropsychiatric disorders including bipolar disorder, major depression, schizophrenia and autism could result from altered epigenetic status. (Van Vliet, Oates, & Whitelaw, 2007)

There are a number of reasons to support the hypothesis that epigenetic effects may also underlie the initiation, maintenance or pathogenesis of smoking. For example, with respect to initiation, adoption studies have demonstrated that early gene-environment interaction (GxE) effects make prominent contributions to the subsequent development of substance use.(Yates, Cadoret, & Troughton, 1998) Although the biological basis for this increased vulnerability could result from structural changes in the brain that increase the propensity to use in the future, an alternative possibility is that epigenetic changes also may underlie a portion of the increased vulnerability. With respect to maintenance of smoking, changes observed as a consequence of smoking cessation at critical catabolic genes, such as MAOA, may be responsible for some of the withdrawal phenomena noted in smokers undergoing substance use treatment.(R. A. Philibert et al., 2010a) Finally, the demonstration that risk for many smoking-related disorders such as CAD and stroke gradually decrease after smoking cessation suggest the possibility that epigenetic phenomena may underlie a portion of the increased smoking associated risk.(U.S. Department of Health and Human Services, 1990) Hence, there is ample reason to that smoking may be associated with alterations in methylation status.

The Effect of Smoking on DNA Methylation

Building on our earlier work at the serotonin transporter suggesting that methylation may underlie biological basis of some GxE effects,(R. Philibert et al., 2007) our group began to investigate the relationship of smoking to methylation at monoamine oxidase A (MAOA). MAOA was an attractive candidate gene for these initial studies. For example, for many years, it has been known that smoking inhibited brain monoamine oxidase activity and directly inactivated monoamine oxidase activity in saliva.(Fowler, Volkow, Wang, Pappas, Logan, MacGregor, et al., 1996; Fowler, Volkow, Wang, Pappas, Logan, Shea, et al., 1996; Yu & Boulton, 1987) In addition, GxE effects at MAOA have been noted for clinical syndromes associated with substance use.(Kim-Cohen et al., 2006; Prom-Wormley et al., 2008) In 2008, using DNA from lymphoblasts and single locus sequencing technologies, we demonstrated that methylation at two promoter associated CpG islands in MAOA was lower in DNA prepared from those who smoked and that this altered methylation was MAOA genotype dependent.(R. A. Philibert, Gunter, Beach, Brody, & Madan, 2008) Similar findings were shown shortly thereafter at monoamine oxidase B (MAOB) by Launay and colleagues (Launay et al., 2009) which was then followed by confirmation and extension of our prior 2008 results into DNA from whole blood(R. A. Philibert et al., 2010b).

Beginning in 2009, these more local investigations were augmented by the introduction of increasingly more comprehensive systematic platforms such as the initial study by Breton and colleagues using a Illumina Golden Gate platform that interrogated over 1500 residues from 807 genes and which implicated differential methylation of AXL and PTPRO of buccal DNA in response to smoking.(Breton et al., 2009) In 2011, Breitling and colleagues used the Illumina 27K platform which examines over 8000 loci and demonstrated that methylation at F2RL2 (factor II (thrombin) receptor-like 3) in lymphocyte DNA was associated with smoking status.(Breitling, Yang, Korn, Burwinkel, & Brenner, 2011) In 2012, the introduction of the Illumina 450K platform, which examines 485,577 CpG residues and covers 20216 transcripts, potential transcripts or CpG islands, was then used by us to show

the smoking associated demethylation of the aryl hydrocarbon receptor repressor (AHRR) in lymphoblast DNA. Since that time, there have been at least four other genome wide investigations of the role of smoking in a variety of DNA preparations from newborns, new onset smokers and older smokers. These studies have demonstrated that smoking has a progressively pervasive effect on genome by methylation (Breitling, Salzman, Rothenbacher, Burwinkel, & Brenner, 2012; Joubert et al., 2012; R. A. Philibert, Beach, & Brody, 2012; Shenker et al., 2012). In order to more effectively convey a developmentally informed understanding of these progressive effects on DNA methylation and general health status, in remainder of this chapter, we will focus on our studies of African-American subjects with reference to the studies of others where appropriate.

Although individuals can begin smoking at any portion of their life, the vast majority of smokers initiate their use in early adolescence. (Shadel & Cervone, 2011) The pathway to regular daily use can be visualized as occurring in four discrete stages of variable length. Smokers first experience initial exposure (i.e. first cigarettes), followed by experimental smoking (irregular periods of smoking), then by regular smoking (recurrent smoking in specific contexts), and then finally dependent smoking (smoking driven by physiological or psychological cues) (Shadel, Shiffman, Niaura, Nichter, & Abrams, 2000). The risk for severe medical complications is also stage dependent. In general, only individuals with underlying pulmonary disorders are likely to experience adverse health outcomes on the initiation of smoking. However, with continued smoking comes a substantially increased risk for a large variety of complex medical disorders regardless of initial health status. However, even in the presence of severe medical illness, once fully established, smoking behaviors are fairly stable though most smokers experience multiple cycles of quitting and relapsing even if they finally achieve complete abstinence (Kahn & Pearlman, 2006).

The initiation of smoking does not occur in isolation but generally occurs in the context of other externalizing behaviors such as drinking or unsafe sexual practices. (Gerrard, Gibbons, Stock, Lunde, & Cleveland, 2005) Therefore, in efforts to minimize the occurrence of associated clinical phenomena and treat smoking during a more tractable period, many investigators have concluded that intervening in the initial portion of the smoking trajectory may be the most cost-effective mechanism of preventing smoking. Unfortunately, because of the unreliability of self-report data, identifying potential subjects for treatment is problematic.

In an effort to better characterize this initial period of smoking and to discern whether DNA methylation changes were evident at this stage, our group examined the relationship of smoking to genome wide methylation in a group of 399 19-year-old African-American participants in the Strong Healthy African-American Families Project (SHAPE), which is a longitudinal assessment of the effect of health behaviors in rural African-American families. (R. A. Philibert, Beach, et al., 2012) Consistent with prior epidemiological studies which show that African-Americans begin smoking later in life and that males tend to smoke earlier in life than females, male subjects reported more frequent smoking than female subjects (39% vs 17%, $p < 0.01$).

Using the self-report, we analyzed the relationship of genome wide methylation to smokers status in the cohort of 181 males using a three class analytic approach that included non-smokers (n=111), smokers who report less than a ½ pack year (n=42) and those who report greater than or equal to 1 pack year of smoking history. Table 1 delineates the five most significant associations. consistent with our previous genome wide analysis, two probes from AHRR, including cg05575921, were among the most highly associated probes. The effect at these loci was dose-dependent with the overall difference in methylation between smokers and non-smokers being approximately 6% (88% vs 82%).

AHRR demethylation is a logical choice as an early response element for smoking. The aryl hydrocarbon pathway regulates the cytochrome P450 catabolism (CYP1A1, CYP1A2 and CYP1B1) of xenobiotics such as polyaromatic hydrocarbons (PAH) and dioxins.(Nguyen & Bradfield, 2007) The aryl hydrocarbon receptor initiates the induction of this process after binding one of these toxins to its PAH domain. Following binding, this nuclear receptor dimerizes with the aryl hydrocarbon nuclear translocator (ARNT) and translocates to the nucleus where it binds and activates promoters of genes critical to toxin metabolism such as CYP1A1. Normally, this pathway is relatively inactive and is in part kept inactive by the competitive antagonism of AHRR for ARNT or by the steric inhibition of AHR action by AHRR/ARNT dimers at critical DNA motifs of genes in the xenobiotic catabolism pathway. Hence, it is logical to find that alterations in AHRR methylation are associated with exposure to the PAH and dioxins in smoke. However, the exact relationship between AHRR demethylation and induction of the of P450 pathway is not yet fully evident because of the complexity of AHRR, which has at least 21 known splice variants and 10 protein isoforms, and because the areas being de-methylated are within the gene body and intronic (cg05575921 is located in intron 3).

Although it appears that AHRR is the most sensitive locus found yet to date, changes in other loci are still evident though at a less significant level. For example, in our study, methylation at the F2RL3 was different in the smokers versus non-smokers at the nominal level. However, after correction for multiple comparisons, these changes were not significant at the genome wide level. This is true for at least some markers of inflammation. Despite the fact that smoking is known to mediate some of its effects through inflammatory processes, at this stage of exposure, there were no significant differences between cases and controls with respect to CRP levels (unpublished data).

In work under consideration elsewhere, we show that the significance of the AHRR associated methylation signature continues to increase as a function of continued smoking in slightly older African-American subjects (i.e. 22 year old).(R. Philibert, Beach, Li, & Brody, in submission) For example, in the slightly older subjects, the degree of differential demethylation associated with smoking at cg05575921 is increased as compared to the younger smokers (10% in the 22 year olds vs 6% in the 19 yr olds), however genome wide significant demethylation continues to be confined to AHRR. In contrast to the younger subjects, CRP levels are significantly higher in the smokers versus non-smokers (4.6 ± 7.8 mg/dL vs 1.9 ± 2.9 mg/dL) which suggests that smoking associated inflammatory processes may be in play, and providing a potential trigger for the next wave of methylation changes.

By the time 20 years of smoking history has lapsed, the epigenetic remodeling began at AHRH has reached breathtaking proportions. Using biomaterials prepared from a large cohort of African-American adults discordant for smoking status, we have recently examined the relationship between smoking status and DNA methylation using the Illumina HumanMethylation450 Beadchip platform described above (Dogan et al., in submission). In brief, we found that in contrast to that of the younger smokers, the methylome of the adult smokers, almost all of whom had smoked for 20 or more years, was greatly altered. At genome wide significance levels, the methylation status at over 2600 discrete CpG residues were significantly altered. When examined at the nominal significance level, almost 20% of the genome demonstrated evidence of remodeling.

Consistent with our previous observations, the demethylation observed at cg05575921 continues to increase with the average change being approximately 15% (Figure 3). Taken in conjunction with our prior observations, this suggests that methylation status at this locus could be used as a tool to determine both current and cumulative exposure to tobacco smoke. However in addition, a number of loci involved in inflammation and cell death identified in prior communications by other groups including Myo1G, F2RL3 and others show prominent effects. In contrast to the health status of the younger cohorts, the adults who smoke in this study have significant higher rates of inflammatory disease, type II diabetes and other illnesses associated with smoking. Therefore, it is likely that the methylation status of many these other markers is also associated with the secondary health outcomes and that further examination of the relationship of their status to health outcomes could lead to better biomarkers for wide variety of smoking related outcomes.

Clinical Applications of DNA Methylation Technologies

A frequent comment made by critics of basic science studies of substance use is that the results of these more mechanistic studies seldom have impact on the diagnosis, care and treatment of real-world patients. This may not be the case for the analyses of the epigenetic effects of substance use on the human at the genome. Conceivably, the type of approaches described above could lead to the improvement of both pharmacological and prevention interventions for smoking.

The development of better chemotherapeutic approaches is perhaps the more distant horizon. This is not secondary to concerns that substance use does not have an effect on the genome. The premise that epigenetic changes occur as part of the pathogenic process of smoking and other forms of substance use is becoming generally accepted. For example, histone modifications in association with the use of cocaine have been described by a number of investigators.(Kumar et al., 2005) Alcohol use is also associated with changes in DNA methylation.(Manzardo, Henkhaus, & Butler, 2012; R. A. Philibert, Plume, Gibbons, Brody, & Beach, 2012) Taken as a sum, these and other studies leaves little doubt that smoking has substantive, pervasive effects on the genome.

Rather, the reason for the delay in the above information to produce better pharmaceuticals for the treatment of substance use is that the number of agents in current cupboard and developmental pipeline for agents that can specifically target critical sites in the epigenetic machinery of the cell is relatively limited. For example, although many drugs currently used

in neuropsychiatric illnesses such as valproic acid have been about the size to have effects on DNA methylation, the exact magnitude and specificity of these effects are uncertain. (Detich, Bovenzi, & Szyf, 2003) Certain other agents used for other indications are potentially more promising. For example, at low doses (10^{-5} M), theophylline, a purinergic agonist commonly found in tea and used to treat asthma, has inhibitory effects on histone deacetylation. (Ito et al., 2002) Unfortunately, the selectivity of theophylline for the various histone deacetylases (HDAC 1, 2 and 3) is uncertain and at higher concentrations theophylline serves as a potent activator of purinergic (P1) receptors.

An additional complication for attempts to target the epigenetic machinery of the cell is that in contrast to many other sites for pharmaceutical manipulations such as dopamine receptor antagonists, the vast majority of potential epigenetic targets are expressed in every cell of the body and loosely distributed throughout the nucleus. Therefore, it is likely that even if agents highly specific for a specific HDAC, methyl DNA binding protein or histone phosphorylase can be identified, the degree of off target effects of the drug will be substantial enough to preclude clinical use. However, at the same time, it is important to note that our understanding of the exact biomolecules/proteins involved in the initiation, maintenance and remodeling of epigenome is still in its infancy and our ability to encapsulate and deliver medications to discrete cell types is continuously increasing. Consequently, it is highly likely that in the future some efforts to develop clinically useful modifiers of epigenetic activity will succeed.

At present, the most concrete application of epigenetic technologies in the field of substance use will be to increase the sensitivity and specificity of clinical status. For example, with respect to smoking, it may be possible to use DNA methylation assessments described in our 2012 communication to more effectively identify adolescents early in the tobacco use trajectory. This is potentially highly valuable from the public health point of view. Although it is not commonly appreciated, most smokers pass through several discrete phases of smoking including initial exposure, experimental smoking, and intermittent smoking before assuming regular dependent smoking. (Shadel & Cervone, 2011) During these earlier phases of smoking, adolescents have not become permanently entrained by the effects of nicotine withdrawal and are more amenable to behavioral smoking interventions. Perhaps even more importantly, because smoking by adolescents is illegal and is associated with other potentially damage behaviors such as binge drinking and risky sex, by developing DNA methylation tools to more readily identify individuals during this critical period, it may be possible to not only avert regular dependent smoking but many of the unfortunate consequences of the accompanying externalizing behaviors.

A second possible clinical application of this technology is to aid in the more effective treatment of regular smokers. This type of effort is desperately needed. Despite a significant investment in pharmaceutical development and treatment infrastructure, the success of smoking cessation in general clinical population is only 10%. (Anczak & Nogler, 2003; Messer et al., 2007) Critically, from the socioeconomic perspective, smoking cessation is perhaps the single most effective general healthcare intervention available. Therefore, a small increase in the success rate of smoking cessation therapy could have a huge socioeconomic impact.

Currently, three types of pharmacological approaches are generally used in the treatment of smoking, bupropion, varenicline, and nicotine replacement treatments (NRTs). (Herman & Sofuoglu, 2010) The frequency of their use differs. Secondary to concerns about the untoward side effects of varenicline and bupropion, most patients are treated using a NRT, such as the nicotine patch or gum, as the primary or augmenting therapy. The net result of this, as alluded to previously, is that virtually all patients undergoing smoking cessation therapy have high serum cotinine levels whether they are smoking or abstinent. This is unfortunate because it obviates the clinically sensitive tool that is currently available for assessing the success of smoking cessation treatment.

The data discussed here and elsewhere suggest the possibility that assessments of DNA methylation could be used to both qualitatively and quantitatively measure the effectiveness of smoking cessation therapy. For example, by measuring the amount of change in DNA methylation, it may be possible to develop a clinical algorithm that describes the degree of success that individual patients have having quitting smoking. This could increase the rate of smoking cessation treatment in two ways. First, a number of studies have shown that feedback demonstrating the degree of success of smoking cessation has a significant effect on the success of treatment. Similar to the role of a scale in the dietary treatment of obesity, the assessment of DNA methylation could be used to show patients quantifiable results. Second, and perhaps equally valuable, the more rapid ascertainment of treatment failure could help clinicians focus on those patients willing to engage but not succeeding. A troubling phenomenon common to many dialogs in healthcare is the reluctance of patients to share information indicating that treatment has not been effective. Often, this results from a patient needing approval from their care provider and not being seen as a failure. This problem is not unique to smoking cessation therapy and is actually seen in the care of a broad range of clinical illnesses such as diabetes, hypertension and depression. As a result of his failure to engage in truthful dialogue, both valuable time and clinical willingness of patients is lost. The development of a tool that unambiguously identify treatment failures could allow clinicians to focus on critical issues such as the cost of medications or unwanted side effects that impede successful treatment.

Could epigenetic technologies be used to prioritize treatment recommendations? At the current time, this is unclear. At the current time, several potential barriers to the development of DNA methylation technologies for the selection of smoking cessation treatments exist. These include the aforementioned difficulty in ascertaining who is actually responding and the uncertain relationship between peripheral DNA methylation and methylation status in the critical role of substrates being targeted by pharmacotherapy. Recent work by Volkow and associates demonstrates that the smoking associated DNA methylation changes at monoamine oxidase A (MAOA) observed in our earlier studies are associated with changes in central MAOA activity. (Shumay, Logan, Volkow, & Fowler, 2012) Furthermore, recent studies by Mills and associates have shown substantial conservation of Inter-individual differences in DNA methylation. (Mill et al., 2011) Therefore, it is certainly possible that by profiling the DNA methylation signature, we may be able to choose the most effective medication for a given patient. However, before this can come to clinical fruition, substantial amounts of clinical research are needed.

Conclusion

In summary, the advent of methods to assess DNA methylation status has led to substantial increases in our understanding of the mechanisms through which smoking transmits its devastating effects on the biome. These effects are dose and time dependent. At the same time, these advances also present the possibility that clinical tools to increase the effectiveness of clinical screening and smoking cessation therapy can be developed. Because smoking has such a devastating clinical and socioeconomic impact, studies to exploit that possibility are desperately needed.

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Figure 1.

The link between genome methylation and decreased gene expression. DNA methylation is mediated by a family of proteins referred to as DNA Methyltransferases (DNMT 1, 3A, 3B and 3L). Depending on cellular context, these DNMT can transfer methyl groups from methyl donors to CpG residues throughout the genome, including in promoter associated CpG islands. Areas with increased amounts of methylation then attracts a cascade of other proteins, which in turn bundle DNA into tightly packed, transcriptionally inactive chromatin resulting in decreased gene expression from that region.

Figure 2.

The role of AHRR in moderating activity in the xenobiotic detoxification pathway. In the unstimulated state, the aryl hydrocarbon receptor (AHR) is found in the cytoplasm bound to a dimer of heat shock proteins (HSP90) and a small group of auxiliary proteins. Poly aromatic hydrocarbons (PAH) and dioxins from cigarette smoke activate the xenobiotic pathway by diffusing into the cell and binding to the PAH domain of AHR. This causes a disassociation of AHR from its binding partners and in turn, heterodimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT). This dimer pair is then transported into the nucleus where it binds to 5 bp DNA motif found immediately upstream of the translation start site of genes critical to the catabolism of xenobiotics. AHRR, whose transcription is induced by AHR, provides negative feedback by competing with AHR for ARNT and by sterically blocking access of AHR to critical xenobiotic pathway genes.

Figure 3.

The dose and chronological dependency of DNA methylation at AHRR residue cg05575921 as a function of smoking in three cohorts of subjects age 19 (n=181), 22 (n=107) and 50 years old (n=111). As individuals continue to smoke the difference between methylation at this locus continues to grow from approximately 6% in the 19 year olds to over 15% in the 50-year-old subjects. Please also note that the average methylation at this locus continues to decrease even in non-smokers.

Table 1
The Five Most Significantly Associated Probes in DNA from Male 19 Yr Old African Americans

Probe ID	GENE	Average Beta Values for Each Usage Class				uncorrected	
		NS	< ½ PY	1 PY	T-test	FDR	P-value
cg05575921	AHRR	0.878	0.829	0.772	5.65 E-13	2.74E-07	
cg17268033	TUBB8	0.529	0.557	0.581	5.14 E-07	0.12	
cg23576855	AHRR	0.719	0.675	0.652	4.86 E-06	0.55	
cg11620896	MUS81	0.057	0.053	0.051	6.94 E-06	0.55	
cg01060358	RNASEH2B	0.155	0.148	0.145	7.05 E-06	0.55	

* PY = pack year, size for non-smoking (NS) =111, < ½ PY group n=42, 1 PY n=28.