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Review

Alcohol and the methylome: Design and analysis considerations for research using human samples

Nicole Harlaar*, Kent E. Hutchison

University of Colorado Boulder, Boulder, CO 80309-0345, USA

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ABSTRACT

Background: A growing number of studies in human samples have sought to determine whether chronic alcohol use and alcohol use disorders (AUDs) may be associated with epigenetic factors, such as DNA methylation. We review the extant literature in light of some of the challenges that currently affect the design and interpretation of epigenetic research in human samples.
Method: A literature search was used to identify studies that have examined DNA methylation in relation to alcohol use or AUDs in human samples (through July 2013). A total of 22 studies were identified.
Results: Associations with quantitative or diagnostic phenotypes of alcohol use or AUDs have been reported for several genes. However, all studies to date have relied on relatively small samples and cross-sectional study designs. Additionally, attempts to replicate results have been rare. More generally, research progress is hampered by several issues, including limitations of the technologies used to assess DNA methylation, tissue- and cell-specificity of methylation patterns, the difficulties of relating observed methylation differences at a given locus to a functional effect, and limited knowledge about the molecular mechanisms underlying the effects of alcohol on DNA methylation.
Conclusions: Although we share the optimism that epigenetics may lead to new insights into the etiology and pathophysiology of AUDs, the methodological and scientific challenges associated with conducting methylomic research in human samples need to be carefully considered when designing and evaluating such studies.

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1. Introduction

The onset and persistence of alcohol use disorders (AUDs) depends on several factors: inherited predisposition, the environment, including exposure to alcohol itself, and neurobiological changes that take place in response to chronic alcohol use. There

* Corresponding author at: Department of Psychology and Neuroscience, University of Colorado Boulder, 345 UCB, Boulder, CO 80309-0345, USA.
Tel.: +1 303 492 8662; fax: +1 303 492 2967.
E-mail address: nicole.harlaar@colorado.edu (N. Harlaar).

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is currently great interest in the potential role of *epigenetics* in this interplay of factors. Epigenetics refer to mitotically stable molecular processes that regulate gene activity and gene expression without altering the DNA sequence itself (Skinner et al., 2011). Although there is some debate about the processes that may be classed as epigenetic (e.g., Ptashne, 2013), it is generally accepted that they include DNA methylation, non-coding RNAs (ncRNAs), and histone modifications (Tammen et al., 2013). These marks interact with and modify *chromatin*, the protein complex that organizes DNA, and potentially alter the extent to which genes are accessible to *transcription factors*, the regulatory proteins that bind to specific DNA sequences.

Epigenetics has kindled excitement because at least some epigenetic marks are responsive to environmental factors, including drugs of abuse (Feil and Fraga, 2012). Animal work has provided robust evidence for epigenetically mediated changes in gene expression following drug exposure, reflecting the actions of transcription factors such as ΔFosB, cAMP-response element-binding protein (CREB), and neuropeptide Y (NPY; Starkman et al., 2012). These changes may ultimately result in functional alterations of critical brain circuitry implicated in addition (Nestler, 2013). As a result of such findings, the National Institute on Alcohol Abuse and Alcoholism (NIAAA) highlighted a need for further research on the role of epigenetic effects in AUDs in their 5-year strategic plan, "Across the Lifespan" (2009–14). However, concerns about the "seductive allure" of epigenetics have also been expressed (Miller, 2010). In a recent review, Heijmans and Mill (2012) highlight seven issues, or "plagues," that currently beset population-based epigenetic studies of human samples. The goal of this review is to highlight some of these issues in the hope that the design and interpretation of ongoing and future human epigenetic studies on AUDs may be strengthened. We focus specifically on *DNA methylation*, which is the most robust and readily measured epigenetic modification. The interested reader is referred elsewhere for reviews of epigenetic research on AUDs conducted in animals and post-mortem brain samples (Nestler, 2013; Starkman et al., 2012; Wong et al., 2011).

2. Background and conceptual framework

DNA methylation refers to the covalent addition of a chemical tag called a *methyl group* to the 5′ carbon on cytosine. This reaction is catalyzed by DNA methyltransferase (DNMTs) and usually occurs in the context of cytosine-guanine (CpG) dinucleotides. There are roughly 28 million CpG sites in the human genome, which collectively comprise the *methylome* (Eckhardt et al., 2006). The best-characterized methylation target sites are those in CpG islands (CGI), discrete CpG-rich regions, typically located within 1 kb of transcription start sites (TSS), that account for around 10% of CpG sites in the genome. CGIs are usually unmethylated in normal (i.e., non-neoplastic, non-senescent) cells, which permits gene transcription and expression. Observed patterns of DNA methylation in other genomic contexts, such as within the gene body or at enhancer regions, appear to be more nuanced and their functional significance is less well-understood (Jones, 2012; Schübeler, 2012). In disease states, methylation patterns are often disrupted. For example, CGIs in gene promoters may become methylated, which has a repressive effect on gene transcription (Jones, 2012).

The relationships among DNA methylation, alcohol use, and AUDs are likely to be complex. In the animal literature, two scenarios have received particular attention. First, alcohol consumption, as an environmental exposure, could directly alter DNA methylation patterns (Fig. 1A). This scenario considers alcohol use as a casual factor and altered methylation as an outcome. Consistent with this hypothesis, it has been shown that prenatal alcohol exposure leads to changes in methylation in the developing hippocampus (e.g., Chen et al., 2013; Otero et al., 2012). A second scenario posits that methylation is an intermediate step on the causal pathway to disease. That is, certain risk factors lead to the development of AUD symptoms by inducing epigenetically mediated changes in gene expression (Fig. 1B). Consistent with this 'mediating mechanism' hypothesis, work in rodents has shown that the anxiolytic effects of alcohol exposure may be due to histone modifications that lead to chromatin remodeling in the amygdala (Pandey et al., 2008). Risk factors other than alcohol exposure may also be important. For example, genetic variation, including single-nucleotide polymorphisms (SNPs) associated with alcohol dependence risk (e.g., Taqi et al., 2011), sometimes has a substantial impact on methylation patterns, with potential downstream effects on transcription and phenotypic variation.

Research on drugs of abuse such as cocaine (e.g., Damez-Werno et al., 2012; Maze et al., 2010) and amphetamine (Renthal et al., 2008) suggest that some epigenetic effects may be less direct. For example, early drug experience may epigenetically prime certain genes (Robison and Nestler, 2011; Nestler, 2013). These genes do not initially show any changes in expression, but their 'inducibility' is altered, such that they are more likely to be expressed following subsequent drug use (Fig. 1C). Other genes show the reverse pattern, 'desensitization'; they are activated following early acute drug exposure, but not by later exposure (Nestler, 2013; Fig. 1D). It may also be the case that some epigenetic changes occur as a secondary effect of AUDs, perhaps as a result of intracellular signaling cascades (Nestler, 2013; Fig. 1E).

Not all of these scenarios can be readily addressed in research using human samples because opportunities for experimental work are more limited. A typical starting point, therefore, is to investigate whether alcohol use or AUD-related phenotypes are correlated with variation in methylation (Fig. 1F). Work in human postmortem brain samples has provided support for this scenario, showing, for example, that the brains of alcoholics are less methylated overall compared with controls (e.g., Ponomarev et al., 2012). This scenario is fundamentally descriptive in nature; it provides no insight into causal or explanatory pathways. Nonetheless, robust evidence for an association may pinpoint particular loci for further investigation, as well as providing a basis for further research on direction of effect.

3. Literature review

Against this background, we consider the results of published studies that examined DNA methylation in relation to alcohol use or AUDs in human samples. We conducted a literature search on PubMed through 25 July 2013 with the terms "DNA methylation" AND "alcohol" AND "humans." We excluded studies of cancer patients or post-mortem human brain cases. A total of 22 studies were identified, including 15 studies that reported on alcohol-dependent (AD) cases and matched controls, and seven studies that examined the effects of alcohol use in population-based samples. Although methylation is a binary phenomenon (each cytosine base can only be methylated or unmethylated), the methylation state measured at a CpG site for a tissue sample from one individual is an average over cells and alleles. Consequently, all studies treated DNA methylation as a continuous measure (typically a percentage, representing 0–100% methylation). Where multiple tests were conducted but only unadjusted *p* values were reported, we also calculated an FDR *p* value (Benjamini and Yekutieli, 2001). All of the studies that we identified were cross-sectional.

We may divide the studies into three groups. The first group consists of four studies that used proxy markers of 'global' methylation levels in the genome. The largest of these studies combined