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Methylation of a CpG Site Near the ALDH1A2 Gene is Associated with Loss of Control Over Drinking and Related Phenotypes

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Abstract

Background: The pathophysiology of alcohol use disorders (AUDs) may be influenced by epigenetics processes such as DNA methylation, but the identification of DNA methylation patterns associated with AUDs has largely been limited to a handful of candidate genes.

Methods: Participants were hazardous drinkers from the local community (n = 309). All participants completed a baseline clinical interview in which they reported on their loss of control over drinking. A subsample participated in an ethanol (EtOH) infusion experiment (n = 50). DNA was extracted from saliva samples and assayed on the Illumina Infinium HumanMethylation27 DNA Analysis Bead-Chip.

Results: We identified significant associations between loss of control over drinking and DNA methylation at multiple CpG sites. In follow-up analyses of one of our top results, a CpG site near the *ALDH1A2* gene, we found that methylation was negatively associated with rate of intoxication and self-reported feelings of intoxication, consistent with the view that DNA methylation at *ALDH1A2* may be associated with changes in alcohol metabolism.

Conclusions: While these findings require replication, they provide evidence that DNA methylation at multiple CpG sites is associated with loss of control over drinking. It may be useful to examine DNA methylation patterns using several related phenotypes to establish the biological coherence of results and to help prioritize markers for further study.

Keywords

DNA Methylation; Hazardous Drinking; Alcohol Use Disorders; Failed Control

ANIMAL RESEARCH HAS provided intriguing evidence that both vulnerability to drug addiction and drug-induced neural plasticity may partly involve epigenetics: mitotically stable regulatory process that mediate the transcription of genetic information from DNA to RNA, thereby potentially influencing the expression of a given phenotype (Moonat et al., 2010). These changes take place without altering the DNA sequence itself. There has been growing interest in trying to identify specific epigenetic markers associated with drug use and addiction, including alcohol use disorders (AUDs) (Starkman et al., 2012). The current

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study seeks to contribute to these efforts by examining 1 type of epigenetic process, DNA methylation, in a community sample of high-risk drinkers.

DNA methylation involves the addition of a methyl group at position 5 of the cytosine pyrimidine ring in CpG dinucleotides, where cytosine is immediately followed by guanine in the 5' to 3' direction. DNA methylation occurs in concert with other types of epigenetic processes, involving the actions of noncoding RNAs and modifications to histones (Tammen et al., 2013) To date, however, studies with human samples have focused almost exclusively on DNA methylation because DNA samples are relatively stable and are amenable to high-throughput analysis (Kit et al., 2012).

Many studies, including the current work, focus on DNA methylation at CpG-rich regions called “CpG islands” (CGIs). CGIs are overrepresented at active promoter regions and are typically unmethylated, which allows gene transcription to occur (Jones, 2012). Disease states, including AUDs, are often associated with aberrant methylation patterns. For example, CGIs in gene promoters may become methylated, leading to transcriptional inactivation. CpG sites may also become hypomethylated or demethylated, which, depending on the location of the CpG site within the genome, may also have deleterious consequences. The precise mechanisms by which alcohol or other drugs of abuse may alter DNA methylation are currently unclear (Nestler, 2013). They may include dysregulation of the one-carbon metabolism cycle by heavy alcohol use (Fowler et al., 2012; Kruman et al., 2012), or alcohol-induced damage to DNA (e.g., DNA doublestrand breaks; O’Hagan et al., 2008).

Early methylation studies of AUDs in human samples took a “candidate gene” approach, focusing on DNA methylation at genes known to have biological relevance to AUDs. Significantly elevated methylation levels in alcohol dependent (AD) cases compared with controls have been reported for several genes associated with reward processing, including the alpha synuclein (*SNCA*) gene (Bönsch et al. 2005), the μ-opioid receptor gene (*OPRM1*) gene (Zhang et al., 2012), and the dopamine transporter (*DAT*) gene (Hillemacher et al., 2009). An alternative and more recent approach is to use microarrays that target many (~364 to over 450,000) CpG sites (Rakyan et al., 2011). Mirroring research on methylation at “candidate genes,” several array-based studies have identified associations between DNA methylation and AUDs at genes in biological pathways implicated in alcohol dependence (Harlaar and Hutchison, 2013). For example, European American AD cases showed significantly greater methylation levels at the *HTR3* (5-hydroxytryptamine [serotonin] receptor 3A) gene compared with controls (Zhang et al., 2013a). A second study identified numerous methylation differences between Chinese sibling pairs discordant for AD, including elevated methylation levels at the promoter region of gamma-aminobutyric acid A receptor (GABRP) in AD cases (Zhao et al., 2013). In a third example, Chinese AD cases showed significantly lower levels of methylation compared with controls at CpG sites in several alcohol dehydrogenase (ADH) genes and cytochrome P450 2A13 (Zhang et al., 2013b). It is important to note that results have not always been completely clearcut; for example, several candidate gene and array-based methylation studies have reported negative or inconsistent results or have reported associations at genes with unclear biological relevance to AUDs.

In summary, there is a small but growing body of evidence for a role of DNA methylation in AUDs in human samples. However, several issues must be considered. With a few exceptions (e.g., Zhang et al., 2012), sample sizes have generally been small ($n < 200$; Harlaar and Hutchison, 2013). Additionally, there has been relatively little evaluation of potential confounding factors (CFs), notably age (but see Nieratschker et al., 2012). Age-related DNA methylation patterns have been reported in many studies, with CpG sites typically showing increased methylation with age (e.g., Horvath et al., 2012). For studies examining DNA methylation in relation to AUDs, this creates a challenge because most heavy-drinking individuals begin to regularly abuse alcohol as adolescents or young adults (e.g., Hingson et al., 2006).

In the current study, we seek to address some of these issues in an array-based methylation study of harmful drinking levels using DNA from saliva. We took a hypothesis-free approach to identifying DNA methylation markers and followed up one of our top results by examining DNA methylation at that marker in relation to several conceptually relevant measures. By looking for convergence across phenotypes, obtained through different methodologies with different sources of error, observed associations are more likely to have translational significance. To this end, we took advantage of data obtained as part of an ethanol (EtOH) infusion experiment. This experiment provided an opportunity to examine sources of variance in the pharmacokinetics of alcohol, which could include epigenetic factors.

MATERIALS AND METHODS

Sample

A total of 332 participants between ages 21 and 55 were recruited from the Albuquerque metropolitan region by local media advertisements (Claus et al., 2011). Inclusion criteria included drinking at least 5 or more drinks per drinking occasion for men (4 or more for women) at least 5 times in the past month. We excluded respondents if they reported symptoms of psychosis, if they had previously experienced a brain injury or had lost consciousness for more than 5 minutes, or if they currently used drugs other than tobacco or marijuana. We also excluded individuals in need of medical detoxification, as assessed by a score greater than 8 on the Clinical Institute Withdrawal Assessment of Alcohol Scale, Revised (Sullivan et al., 1989). On the day of assessment, participants were required to have a breath alcohol concentration (BrAC) level of 0. Female participants were required to test negative for pregnancy. We excluded 23 individuals for whom we did not have both questionnaire responses and adequate DNA. Thus, our analyses included 309 participants: 216 males (mean age: 31.69 years; SD: 9.50) and 93 females (mean age: 32.54 years; SD: 10.58). The sample consisted of 45% white non-Hispanic, 26.2% white Hispanic/Latino, 5.5% Native American, 1.9% black, 0.6% Asian participants, and 20.7% mixed race individuals. Approximately 54.4% of participants smoked tobacco.

Replication sample: We sought to replicate our top findings in an independent sample ($n = 36$) recruited from the Denver-Boulder metropolitan area for a pharmacological intervention study (reported in Hutchison et al., 2006). This sample consisted of 25 males (mean age:

38.92 years; SD: 9.31) and 11 females (mean age: 44.18 years, SD: 10.25). Approximately 44.4% of participants smoked tobacco.

Clinical Behavioral Assessments

All participants completed a clinical interview during a baseline session. Our primary dependent variable was loss of control over drinking over the past 90 days, which was assessed using the Failed Control (FC) subscale of the Impaired Control Scale (Heather et al., 1998). This measure was selected because our previous work suggested that the FC scale was most strongly associated with cueelicited changes in blood oxygen level dependence response in brain regions known to play a critical role in addictive behaviors (e.g., precuneus and striatum; Claus et al., 2011). The interview also included the Alcohol Dependence Scale (ADS) (Skinner and Horn, 1984), the Timeline Follow-Back for quantity and frequency of alcohol and cigarettes (Sobell and Sobell, 1992), and (discovery sample only) the Alcohol Use Disorder Identification Test (AUDIT) (Babor et al., 2001). Sample characteristics on these measures are shown in Table 1.

Additional Measures

Although the FC scale was our primary measure of interest, we used data from an EtOH infusion experiment to explore additional hypotheses that emerged from the initial analysis. These data were collected on a subset of 50 participants from the primary sample, consisting of 36 males (mean age: 25.69 years; SD: 4.09) and 14 females (mean age: 23.71 years; SD: 2.40). Briefly, an intravenous catheter was placed in a forearm vein of the participant's nondominant arm and kept open using a D5W (dextrose 5% in water) infusion over a period of 2.5 hours beginning at 1 PM on the day of testing. There were 3 target BrACs: 0.02, 0.04, and 0.06. Alcohol was infused over 15 minutes (±1 minute) until the subject reached a BrAC of 0.02. They were maintained ("clamped") at this level for 15 minutes, during which time they completed the Subjective High Assessment Scale (Schuckit, 1980) plus other measures of subjective stimulation, sedation, and mood. This was repeated at the target BrAC levels of 0.04 and 0.06. To ensure that each subject achieved the same BrAC level (±5 mg%) in approximately the same amount of time, the infusion rate for each subject was estimated using physiologically based pharmacokinetic modeling (Ramchandani and O'Connor, 2006) that took into account the participant's weight, height, age, and gender.

DNA Methylation Processing and Analysis

Saliva samples (5 ml) from all participants were obtained during the baseline session. DNA (1 μg) was isolated from these samples using the Qiagen® Puregene DNA prep kit (Qiagen, Valencia, CA) and bisulfite-converted using the Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA). DNA methylation profiling was performed with the Infinium HumanMethylation27 DNA Analysis BeadChip (Illumina Inc, San Diego, CA), which targets approximately 27,578 CpG sites at a single-nucleotide resolution. We focused only on autosomal CpG sites (n = 26,432).

Using the *R* package *FDb.InfiniumMethylation.hg19.db*, Illumina identifiers were mapped to the hg19 genome build. All analyses were conducted in the *R* environment (R Core Team, 2013).

Because prefiltering to remove noninformative probes can increase detection power in microarray experiments (Bourgon et al., 2010). CpG sites with a detection *p*-value (a value representing the measured signal compared with negative controls) over 0.05 were removed from the data. We also removed nonvariable probes, defined as CpG sites with a relative standard deviation <15%. Following these steps, 22,046 CpG sites remained available for analysis. For each CpG site, a β-value is derived, which approximately corresponds to the percentage of methylated DNA molecules in a given sample. However, due to the heteroskedasticity of β-values that causes the extremes to have much lower variability, we used *M*-values to quantify methylation at each CpG site (Du et al., 2010). Signal intensities were quantile-normalized prior to the calculation of *M*-values.

To ensure that any significant results were not due to unmeasured nonbiological variables associated with the batch in which a sample is run (e.g., location of samples on different chips), normalized *M*-values were corrected for batch using the Empirical Bayes method *ComBat* (Johnson et al., 2007; Leek et al., 2012). We used Independent Surrogate Variable Analysis (ISVA) to model both prespecified and unmeasured potential CFs (Teschendorff et al., 2011). ISVA uses an iterative procedure based on independent component analysis that simultaneously estimates biological signal of interest as well as effects of unwanted sources of variability. Gender, ethnicity, and average number of cigarettes per smoking day were included as prespecified CFs. To ensure that true biological signal was not removed, only surrogate variables (SVs) that were significantly associated ($p < 0.05$) with CFs were included.

Pyrosequencing

We sought to verify DNA methylation of our top results in our replication sample using locus-specific pyrosequencing of bisulfite-converted DNA. Prior to treatment, participants provided a saliva sample from which DNA was extracted, bisulfite-converted, and submitted for pyrosequencing. This work was performed by EpigenDx (Worcester, MA) according to standard procedures with a unique set of primers that were developed by EpigenDx. DNA methylation level at each site investigated was expressed as percentage of methylation.

RESULTS

In our initial analysis, ISVA was used to compute associations between quantile-normalized *M*-values and FC scores, adjusting for 1 significant SV. Gender was the only prespecified CF to correlate with the SV ($r = -0.18$, $p = 0.002$). We identified 51 CpG sites that met a false discovery rate (FDR) $p < 0.05$; these CpG sites are listed in full in the Table S1. Because the impact of batch on microarray experiments continues to be an area of active research (Scherer, 2009), we repeated the analyses on nonbatch-corrected data, also with 1 SV that was significantly correlated with gender ($r = -0.18$, $p = 0.001$). A total of 207 CpG sites met an FDR $p < 0.05$, listed in Table S2. Twenty-eight CpG sites were significant at FDR $p < 0.05$ in both lists; these sites are summarized in Table 2(a) (test statistics) and 2(b) (annotation information provided by Illumina) and were advanced to the next stage of analysis. In general, *M*-values were positively correlated with FC scores, indicating that greater methylation levels were associated with poorer control over drinking.

Although we were principally concerned with CpG sites that showed robust associations across both batch- and nonbatch-corrected data in the current study (i.e., the CpG sites in Table 2a and 2b), we also conducted exploratory analyses of the batch- and nonbatch-corrected data using the functional annotation clustering tool in the Database for Annotation, Visualization and Integrated Discovery (Huang et al. 2009). We used the "Genetic association disease" and "Genetic association disease classes" databases to cluster genes associated with the top CpG sites. Options were set to their default values and annotations were accessed as indexed on May 26, 2013. No single cluster was identified for the batch-corrected findings. For the nonbatch-corrected findings, 3 clusters were identified (detailed in Table S3). The top cluster (enrichment score: 1.26) included 4 genes: 5-hydroxytryptamine (serotonin) receptor (HTR2A), dopamine receptor D2 (DRD2), dopamine receptor D5 (DRD5), and neuroepithelial cell transforming 1 (NET1).

Age Effects

Within our sample, there was a significant correlation between FC scores and age (r = 0.47, $p < 0.01$), indicating, as expected, that older individuals were more likely to report difficulties with control over drinking. Because methylation is also known to correlate with age, observed associations between DNA methylation and FC scores may be driven, in part, by age, or by the joint effects of age and drinking. Indeed, when we computed partial correlations between FC scores and methylation values for each of the 28 top CpG sites (significant in both batch-corrected and nonbatch-corrected data), adjusting for age, almost all associations were attenuated (Table 3).

To evaluate the effects of age on the association between FC scores and DNA methylation levels more closely, we used a propensity-score matching (PSM) analysis. PSM was originally developed to reduce the effects of CFs when estimating the effects of treatments when using observational data (Rosenbaum and Rubin, 1983), but this method has also proven to be more useful than other techniques for controlling for background covariates of interest (McCaffrey et al., 2013).

In the current study, the "treatment" was having poorer control over drinking, operationalized as having an FC score above the median (scores ≥ 20; high-FC group) versus below the median (score <20; low-FC group). Generalized boosted regression modeling was used to estimate a propensity score for each individual, defined as the probability of being in the high-FC group based on age, gender, ethnicity, and average number of cigarettes per smoking day. Participants in the high-FC group were then matched (with replacement) to participants in the low-FC group with a very similar estimated propensity score. Eight participants could not be adequately matched and were dropped from the analysis. After matching, t-tests indicated that there were no significant differences in age, gender, ethnicity, and average number of cigarettes per smoking day between the high-FC and low-FC groups. Standardized effect sizes ranged from −0.01 (ethnicity) to 0.15 (age).

In the final step of the PSM analysis, we examined the association between FC scores and batch-corrected DNA methylation values at each of the 28 CpG sites using propensity-score-adjusted generalized linear models, stratified by FC group. These analyses included case

weights corresponding to the propensity scores. As shown in Table 3, 11 CpG sites remained significantly associated with FC scores at FDR $p < 0.05$.

Additional Analyses with a CpG Site at the ALDH1A2 Gene

We selected the CpG site in the *ALDH1A2* gene, cg00930873, for more detailed evaluation. According to annotation information provided by Illumina, this CpG site is located in a CGI at a differentially methylated region, where multiple adjacent CpG sites show differential methylation. We further examined the characteristics of this site using the University of California, Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu; UCSC Genome Bioinformatics, Santa Cruz, CA). Chromatin immunoprecipitation sequencing (ChIP-seq) data generated by the ENCODE Project Consortium (ENCODE project consortium et al., 2012) indicate that this CpG site overlaps with several transcription factor binding sites, notably CCNT2 and E2F4. Moreover, chromatin profiling of ChIP-seq data (Ernst et al., 2011) suggests that the region surrounding cg00930873 may be classed as an active or poised promoter in multiple cell types.

The decision to follow-up on cg00930873 was based on 3 factors: the association emerged using both the batch-corrected and nonbatch-corrected data, it remained significant in the propensity score analysis, and this CpG site is located in a biologically-relevant gene. *ALDH1A2* belongs to the aldehyde dehydrogenase 1 (ALDH1) gene family (Muzio et al., 2012), which is linked to the synthesis of enzymes involved in alcohol metabolism. The *ALDH1A2* gene encodes an enzyme responsible for the synthesis of retinoic acid (RA). Links between retinol and alcohol metabolism have been reported (Napoli, 2011). For example, mice that lack retinoid X receptor α expression in the liver are more susceptible to alcoholic liver disease (Dai et al., 2003; Molotkov and Duester, 2002), and chronic EtOH treatment of rats leads to a reduction in RA levels in liver and serum that may contribute to liver carcinogenesis and tissue damage (Wang et al., 1998). Additionally, 1 study has reported evidence for an association between a haplotype of 6 contiguous single-nucleotide polymorphisms in *ALDH1A2* and maximum number of drinks consumed, a phenotype related to alcohol metabolism capacity (Sherva et al., 2009). Finally, as noted in the Introduction, a recent study reported evidence that some ADH-related genes were epigenetically altered in AD cases compared with controls (Zhang et al., 2013b). Accordingly, a series of analyses were designed to address the question of whether methylation at the *ALDH1A2* site might be associated with measures related to individual differences in alcohol metabolism.

As described in Methods and Materials, 50 participants participated in an EtOH infusion study. Participants received a standardized infusion of EtOH and completed assessments when they reached a target BrAC of 0.02, 0.04, and 0.06. Although the study was designed to control the BrAC, there was variance in how quickly each participant reached the target BrAC. We hypothesized that we would observe an association between methylation levels and the time taken to reach the target BrAC if methylation of the *ALDH1A2* site was associated with metabolism of EtOH. To that end, the 50 participants were divided into high- and low-methylation groups based on the median methylation level at cg00930873 (*M*-value ≥ -3.63 and < -3.63), and we tested whether there were group differences in time to reach

each target BrAC, using a 2 (high- vs. low-methylation group) × 3 (BrAC = 0.02, 0.04, 0.06) analysis of variance. The analysis demonstrated a significant main effect of methylation group such that the high-methylation group reached the target BrAC more quickly, $F(1, 48) = 13.04$, $p < 0.001$, as well as a significant methylation by BrAC interaction, $F(1, 48) = 9.99$, $p = 0.003$ (see Fig. 1A). The high- and low-methylation groups differed most at the BrAC of 0.06. The correlation between methylation level and overall time to reach a BrAC of 0.06 was $r = -0.44$, $p < 0.002$, indicating that it took individuals with higher methylation levels less time to reach target BrAC (i.e., slower rates of alcohol elimination or faster rates of alcohol absorption).

If methylation status of the site near the *ALDH1A2* gene alters some aspect of alcohol metabolism, we hypothesized that we would observe a significant effect of methylation group on the participants' subjective report about how intoxicated they felt at each target BrAC. Indeed, an analysis of subjective intoxication indicated a group by BrAC target interaction such that the high-methylation group reported significantly greater subjective intoxication at a target BrAC of 0.06, $F(1, 48) = 4.62$, $p < 0.05$ (see Fig. 1B). The correlation between *ALDH1A2* methylation and subjective high at a target BrAC of 0.06 was $r = 0.38$, $p < 0.01$, indicating that individuals with higher methylation levels felt more intoxicated at this BrAC level.

Pyrosequencing

We used pyrosequencing to quantify DNA methylation near cg00930873. This CpG site is located at position 58357973 (GRCh37/hg19). In our pyrosquencing experiment, methylation was assessed at 4 CpGs sites 643 to 627 base pairs upstream of the TSS (corresponding to positions 58357990, 58357988, 58357983, and 58357974 in GRC h37/hg19). DNA methylation levels were expressed as a percentage of methylation. Analysis of our replication sample indicated that average methylation levels at the 4 positions were very low (5.6, 5.1, 3.3, and 5.9%). At 58357974, the position with the highest average methylation level and the position closest to our original site of interest, the replication sample, the partial correlation between DNA methylation level at position 58357974 and FC scores, controlling for age, was 0.17 ($p = 0.30$), which is not significantly different from the partial correlation between FC scores and methylation at cg00930873 in our discovery sample (0.13; $z = 0.22$; $p = 0.826$, 2-tailed test).

DISCUSSION

We identified significant associations between DNA methylation at several CpG sites and self-reported loss of control over alcohol use, which is a hallmark of AUDs. Mirroring previous reported studies, these associations were mostly positive, indicating that higher methylation was associated with poorer control over drinking. We also sought to consider the effects of age, which has generally been neglected in the alcohol research literature on DNA methylation. As age is known to be associated with both methylation and alcohol dependence symptoms, it may drive the observed associations between methylation and FC scores. Alternatively, a true association between methylation and FC scores may be hidden or attenuated because both age and FC scores are associated with methylation. In the

current study, a PSM analysis indicated that methylation at 11 CpG sites did not appear to be influenced significantly by age within our sample. Our findings suggest that age likely confounds observed associations between alcohol use and DNA methylation at many CpG sites. Consequently, careful attention must be paid to consider the joint and independent effects of age and alcohol use on atypical methylation patterns in older individuals.

We also examined the extent to which one of our top results was associated with other phenotypes that may be influenced by alcohol-related DNA methylation. Although this approach cannot be used to infer causality of the effects of alcohol use on DNA methylation, marshaling support from other phenotypes may help to provide evidence for the biological plausibility and coherence of our findings. We selected a CpG site (cg00930873) in the *ALDH1A2* gene for further analysis on the grounds that DNA methylation at this gene may be linked with the physical and psychological factors implicated in the absorption and metabolism of alcohol. Consistent with hypotheses, individuals with higher methylation levels at cg00930873 reached target BrAC more quickly and were more likely to feel intoxicated at the highest target BrAC of 0.06. Overall, these findings provide relatively consistent evidence, across multiple measures with different sources of error, for a role of DNA methylation at 1 site in the *ALDH1A2* gene related to alcohol metabolism and alcohol dependence symptoms.

Because this was a cross-sectional study, the top hits, including the CpG site in *ALDH1A2*, must be regarded as preliminary. With regard to *ALDH1A2*, our findings suggest that failure to control drinking and resultant heavy alcohol consumption increases DNA methylation at this gene. Speculatively, increased DNA methylation may lead to reduced levels of RA synthesis and inhibited retinoid signaling that in turn lead to increased acetaldehyde toxicity and sensitivity to alcohol. Although *ALDH1A2* knockout has proven to be lethal in animal models (Niederreither et al., 1997), it would be possible to test the notion that reductions in *ALDH1A2* mRNA lead to increased levels of EtOH and behavioral signs of intoxication by using an RNAi approach to knock down *ALDH1A2* mRNA in an animal model. Data from animal models will be important for examining the downstream effects and mechanisms underlying observed associations between methylation and AD symptoms (Harlaar and Hutchison, 2013).

Our pyrosequencing analyses yielded a positive correlation between FC scores and methylation at the site closest to cg00930873, but this correlation was not significantly different from zero. This likely reflects several reasons, including the small size of our pyrosequencing sample, low variability of methylation levels at the pyrosequenced sites, and assay design limitations, which meant that we were not able to sequence the exact nucleotide position of cg00930873 in the pyrosequencing experiments. Previous studies have reported that the precision of the Illumina platform appears to compare well with other platforms, but independent replication of the current results in a larger sample are highly desirable.

Our study has several other limitations. First, for funding reasons, we were unable to extend our replication efforts to a larger number of our top hits. Although we focused primarily on methylation at the *ALDH1A2* gene, several other results were significantly associated with FC scores and could be considered in future investigations. Second, DNA methylation

at many CpG sites is likely to be tissue-specific. This is problematic for in vivo human research on AUDs because biofluids such as blood or saliva are the only tissue sources that can be easily obtained from large samples. Establishing cross-tissue correspondence of DNA methylation patterns will likely require the triangulation of results across different methodologies, including well-characterized human brain samples as well as animal models. Third, the Infinium HumanMethylation27 BeadChip has limitations. This array primarily targets CGIs in the promoter regions in genes, yet it is increasingly clear that aberrant methylation patterns outside CGIs (e.g., at CGI "shores") and away from promoter regions (e.g., in gene bodies) may also be important for understanding complex traits and diseases (Jones, 2012). Additionally, the Infinium HumanMethylation27 BeadChip only covers 1 or 2 CpG sites per gene. This low coverage precludes the investigation of methylation patterns that occur at a regional level (differentially methylated regions), which may show more robust associations than single CpG sites (Jaffe et al., 2012).

Notwithstanding these limitations, this study extends the nascent field of addiction epigenetics by examining a relatively large sample and taking advantage of multiple quantitative measures of alcohol-related phenotypes to examine the biological validity of one of our top results. Much still needs to be performed in terms of addressing issues such as causal significance and tissue specificity, but continuing research in this area may lead to new insights into the role of DNA methylation in the etiology and pathophysiology of AUDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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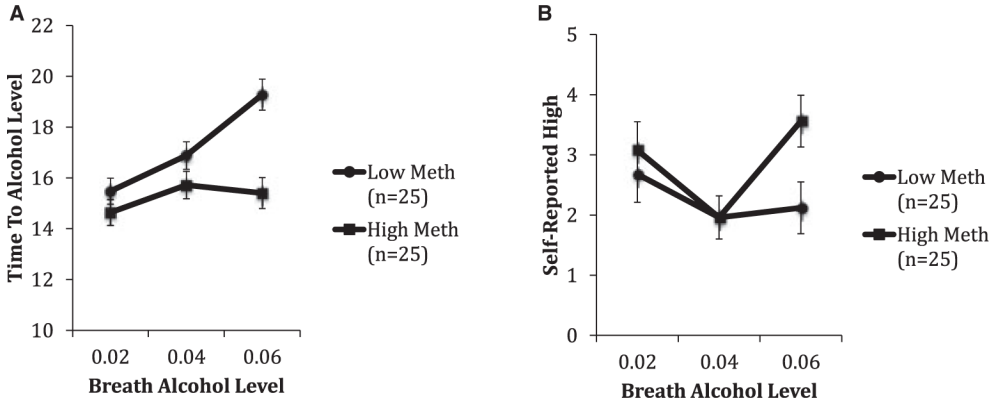

Fig. 1.

Phenotypic differences between participants scoring above and below the median methylation level at cg00930873 for: (1) average time (mins) to reach target breath alcohol concentration (BrAC) levels of 0.02, 0.04, and 0.06 (**A**); and (2) self-reported subjective high at BrAC levels of 0.02, 0.04, and 0.06 (**B**).

Table 1.

Sample Characteristics for the Discovery (n = 309) and Replication (n = 36) Samples

	Discovery sample						Replication sample					
	Full sample (n = 309)		Males (n = 216)		Female (n = 93)		Full sample (n = 36)		Males (n = 25)		Females (n = 11)	
	m	SD	m	SD	m	SD	m	SD	m	SD	m	SD
Age (years)	31.94	9.83	31.69	9.5	32.54	10.58	40.53	9.77	38.92	9.31	44.18	10.25
Failed Control	19.16	9.57	18.64	9.47	20.38	9.75	17.25	6.95	20.32	7.15	18.91	10.72
Average drinks/drinking day	7.32	3.89	7.52	3.79	6.19	3.49	8.5	5.67	9.95	5.99	5.22	3.03
Average cigarettes/smoking day[a]	4.81	7.1	4.7	6.99	5.09	7.4	0.59	0.96	0.67	1.06	0.25	0.8
ADS total	13.4	7.99	12.96	7.65	14.43	8.71	14.91	6.22	14.79	5.49	15.18	7.88
AUDIT total	19.06	7.63	18.98	7.27	19.23	8.4	NA	NA	NA	NA	NA	NA

Failed Control: total score on Failed Control subscale of Impaired Control Scale. AUDIT total: Alcohol Use Disorder Identification Test total score; ADS total: Alcohol Dependence Scale total scores. Average drinks/drinking day and average cigarettes/smoking day were estimated from Timeline Follow-Back interviews.

[a]54.4% of participants in the discovery sample and 44.4% of participants in the replication sample were smokers (at least 1 cigarette in past 60 days).

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Table 2.

(a) CpG Sites Significantly (FDR $p < 0.05$) Associated with Failed Control (FC) Scores: Test Statistics for Batch-Corrected and Nonbatch-Corrected Data; (b) CpG Sites Significantly (FDR $p < 0.05$) Correlated with FC Scores: Annotation Information

		Batch-corrected data			Nonbatch-corrected		
CpG marker	*r*	*t*	*p*	FDR *p*	*t*	*p*	FDR *p*
(a)							
cg14483391	0.30	5.60	4.89E-08	0.001	4.68	4.29E-06	0.003
cg06572160	0.27	4.96	1.15E-06	0.005	5.26	2.71E-07	0.002
cg22971191	0.27	4.96	1.17E-06	0.005	3.81	0.000168	0.024
cg15357639	0.25	4.54	8.09E-06	0.021	4.81	2.43E-06	0.003
cg15344028	−0.25	−4.46	1.14E-05	0.021	−4.84	2.10E-06	0.003
cg04278702	0.24	4.42	1.38E-05	0.021	4.47	1.08E-05	0.005
cg27553955	0.24	4.40	1.53E-05	0.021	5.12	5.31E-07	0.002
cg09656934	0.24	4.39	1.58E-05	0.021	4.98	1.07E-06	0.002
cg14802951	0.24	4.36	1.77E-05	0.021	4.2	3.51E-05	0.011
cg13688966	0.24	4.33	2.04E-05	0.021	4.21	3.42E-05	0.011
cg18601426	0.24	4.32	2.10E-05	0.021	4.42	1.38E-05	0.006
cg13407883	−0.24	−4.27	2.63E-05	0.022	−4.20	3.55E-05	0.011
cg00930873	0.24	4.25	2.87E-05	0.022	4.94	1.26E-06	0.002
cg26614073	−0.23	−4.24	2.93E-05	0.022	−4.57	7.18E-06	0.004
cg24776407	0.23	4.18	3.83E-05	0.027	5.41	1.27E-07	0.002
cg10660256	0.23	4.14	4.55E-05	0.029	3.76	0.0002	0.027
cg06952310	0.23	4.12	4.91E-05	0.029	3.49	0.0006	0.045
cg02506908	0.23	4.11	5.08E-05	0.029	3.98	8.49E-05	0.016
cg05492270	0.23	4.08	5.80E-05	0.031	3.63	0.0003	0.035
cg08209133	0.23	4.07	6.05E-05	0.031	4.37	1.70E-05	0.007
cg17861230	0.22	4.04	6.66E-05	0.033	4.68	4.32E-06	0.003
cg20114394	0.22	3.99	8.32E-05	0.038	3.92	0.0001	0.019
cg26845300	0.22	3.93	0.0001	0.044	4.83	2.19E-06	0.003
cg11314684	−0.22	−3.92	0.0001	0.045	−4.18	3.76E-05	0.011
cg08379517	0.22	3.91	0.0001	0.045	3.53	0.0005	0.042

cg00548268	0.22	3.91	0.0001	0.045	4.37	1.72E-05	0.007
cg21296230	0.21	3.85	0.0001	0.048	4.61	5.95E-06	0.004
cg03355526	0.21	3.83	0.0002	0.048	4.94	1.31E-06	0.002

CpG marker	Chr	Gene	Map coordinate	Location	Relation to CGI (if available)	$\bar{x}$ % meth.	SD
(b)							
cg14483391	3	HTR3D	183749227	TSS1500	–	0.49	0.06
cg06572160	19	KCNC3	50831901	1st Exon	Island	0.11	0.07
cg22971191	13	SLC10A2	103719706	TSS1500	–	0.72	0.08
cg15357639	3	OGG1	9790443	TSS1500	5′ Shore	0.79	0.09
cg15344028	2	ICOS	204801510	5′UTR; 1st Exon	–	0.65	0.06
cg04278702	6	HTR1E	87647399	1st Exon; 5′UTR	Island	0.05	0.01
cg27553955	2	KCNG3	42720326	1st Exon	Island	0.30	0.05
cg09656934	1	TDRD5	179561500	5′UTR	Island	0.32	0.1
cg14802951	9	ADAMTS13	136287409	5′UTR; Body	5′ Shelf	0.62	0.11
cg13688966	3	TM4SF4	149192377	TSS200	–	0.69	0.12
cg18601426	7	PTPRN2	158381220	TSS1500	Island	0.24	0.04
cg13407883	19	SIGLEC9	51627843	TSS1500	–	0.84	0.05
cg00930873	15	ALDH1A2	58357973	TSS200	Island	0.08	0.03
cg26614073	3	SCAP	47517819	TSS1500	3′ Shore	0.56	0.07
cg24776407	11	MYEOV	69061634	5′UTR; 1st Exon	–	0.70	0.11
cg10660256	5	BHMT	78407683	5′UTR; 1st Exon	–	0.10	0.03
cg06952310	19	CSPG3	19327990	Body	–	0.59	0.07
cg02506908	12	HPD	122297408	TSS1500	–	0.80	0.09
cg05492270	11	RNF121	71640085	Body; TSS1500	–	0.09	0.03
cg08209133	4	SLC10A4	48485624	1st Exon	Island	0.16	0.04
cg17861230	19	PDE4C	18343901	Body	Island	0.23	0.06
cg20114394	3	FSTL1	120170441	TSS1500	Island	0.72	0.09
cg26845300	6	SNX9	158243833	TSS1500	Island	0.06	0.02
cg11314684	1	AKT3	244006288	Body	–	0.31	0.08
cg08379517	19	LRRC25	18509505	TSS1500	–	0.59	0.18
cg00548268	7	NPTX2	98245830	TSS1500	Island	0.33	0.06

cg21296230	15	GREM1	33010536	5′UTR	Island	0.13	0.04
cg03355526	5	ZNF454	178368415	5′UTR	Island	0.13	0.06

Annotation information was derived from annotation files provided by Illumina. Chr: Chromosome. Map coordinate is based on GRCh37/hg19. UTR: untranslated region; TSS200: 200 bp within transcriptional start site; TSS1500: 1.5 kb within transcriptional start site. CGI: CpG island; 5′ Shore: CpG site at a CGI “shore” at the 5′ end; 3′ Shore: CpG site at a CGI “shore” at the 3′ end. NA: Information not available. $\bar{x}$ and SD of methylation (for ease of interpretability, these are based on beta values rather than *M*-values, where 0 = no methylation and 1 = complete methylation). FDR, false discovery rate.

Table 3.

Associations Between Failed Control (FC) Scores and DNA Méthylation, Controlling for Age, at the 28 CpG Sites that Were Significantly Associated with Age (FDR $p < 0.05$) in Both the Batch-Corrected and Nonbatch-Corrected Data Analyses

		Partial correlations (n = 309)			PS-adjusted generalized linear model (n = 301)				
CpG site	**Gene**	***r***	***p***	**FDR *p***	***β***	***SE***	***t***	***p***	**FDR *p***
cg14483391	HTR3D	0.17	0.002	0.019	7.09	1.98	3.58	0.0004	0.0057
cg06572160	KCNC3	0.12	0.037	0.099	1.93	0.75	2.57	0.0106	0.0389
cg22971191	SLC10A2	0.15	0.009	0.050	4.30	1.37	3.15	0.0018	0.0154
cg15357639	OGG1	0.07	0.211	0.297	1.28	0.76	1.68	0.0943	0.1467
cg15344028	ICOS	−0.12	0.033	0.099	−3.35	1.77	−1.90	0.0585	0.1170
cg04278702	HTR1E	0.13	0.029	0.099	4.61	1.71	2.69	0.0076	0.0354
cg27553955	KCNG3	0.04	0.453	0.507	3.14	1.99	1.58	0.1150	0.1533
cg09656934	TDRD5	0.12	0.039	0.099	2.07	0.85	2.45	0.0149	0.0415
cg14802951	ADAMTS13	0.07	0.224	0.299	1.56	0.96	1.62	0.1060	0.1533
cg13688966	TM4SF4	0.10	0.092	0.198	1.50	0.74	2.04	0.0424	0.0913
cg18601426	PTPRN2	0.17	0.002	0.019	4.86	2.17	2.24	0.0261	0.0609
cg13407883	SIGLEC9	−0.17	0.003	0.021	−4.68	1.51	−3.09	0.0022	0.0154
cg00930873	ALDH1A2	0.13	0.024	0.096	3.29	1.35	2.43	0.0155	0.0415
cg26614073	SCAP	−0.07	0.212	0.297	−3.66	1.51	−2.42	0.0163	0.0415
cg24776407	MYEOV	0.09	0.119	0.222	1.29	0.86	1.50	0.1340	0.1658
cg10660256	BHMT	0.08	0.160	0.264	4.01	1.57	2.56	0.0111	0.0389
cg06952310	CSPG3	0.14	0.013	0.061	4.64	1.59	2.91	0.0038	0.0215
cg02506908	HPD	0.09	0.119	0.222	0.84	0.91	0.93	0.3540	0.3540
cg05492270	RNF121	0.18	0.002	0.019	6.42	1.73	3.72	0.0002	0.0057
cg08209133	SLC10A4	0.07	0.239	0.304	2.86	1.60	1.79	0.0743	0.1387
cg17861230	PDE4C	0.02	0.783	0.812	1.93	1.32	1.46	0.1450	0.1658
cg20114394	FSTL1	0.05	0.377	0.459	0.99	0.87	1.13	0.2590	0.2686
cg26845300	SNX9	0.08	0.157	0.264	1.84	1.27	1.45	0.1480	0.1658
cg11314684	AKT3	−0.08	0.185	0.288	−1.89	1.29	−1.46	0.1460	0.1658
cg08379517	LRRC25	0.05	0.430	0.502	0.87	0.51	1.70	0.0899	0.1467
cg00548268	NPTX2	0.04	0.474	0.510	2.18	1.57	1.39	0.1650	0.1777

		Partial correlations (*n* = 309)			PS-adjusted generalized linear model (*n* = 301)				
CpG site	**Gene**	***r***	***p***	**FDR *p***	***β***	***SE***	***t***	***p***	**FDR *p***
cg21296230	GREM1	0.01	0.932	0.932	2.09	1.31	1.59	0.1130	0.1533
cg03355526	ZNF454	0.10	0.069	0.161	1.61	0.95	1.70	0.0902	0.1467

Both partial correlations and the results from propensity-score (PS) adjusted generalized linear models are reported. PS derived from high-FC versus low-FC groups (scoring above and below the median on FC scores, respectively), using age, gender, ethnicity, and average cigarettes per smoking day as covariates. All analyses were based on batch-corrected methylation data. FDR, false discovery rate.