Published in final edited form as:

Am J Addict. 2017 August ; 26(5): 502–515. doi:10.1111/ajad.12465.

Review: DNA Methylation and Alcohol Use Disorders: Progress and Challenges

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Abstract

Background and Objectives—Risk for alcohol use disorders (AUDs) is influenced by gene–environment interactions. Environmental factors can affect gene expression through epigenetic mechanisms such as DNA methylation. This review outlines the findings regarding the association of DNA methylation and AUDs.

Methods—We searched PubMed (by April 2016) and identified 29 studies that examined the association of DNA methylation and AUDs. We also evaluated the methods used in these studies.

Results—Two studies demonstrated elevated global (repetitive element) DNA methylation levels in AUD subjects. Fifteen candidate gene studies showed hypermethylation of promoter regions of six genes (*AVP*, *DNMT3B*, *HERP*, *HTR3A*, *OPRM1*, and *SNCA*) or hypomethylation of the *GDAP1* promoter region in AUD subjects. Five genome-wide DNA methylation studies demonstrated widespread DNA methylation changes across the genome in AUD subjects. Six studies showed significant correlations of DNA methylation with gene expression in AUD subjects. Three studies revealed interactive effects of genetic variation and DNA methylation on susceptibility to AUDs. Most studies analyzed AUD-associated DNA methylation changes in the peripheral blood; a few studies examined DNA methylation changes in postmortem brains of AUD subjects.

Discussion and Conclusions—Chronic alcohol consumption may result in DNA methylation changes, leading to neuroadaptations that may underlie some of the mechanisms of AUD risk and persistence. Future studies are needed to confirm the few existing results, and then to elucidate whether DNA methylation changes are the cause or consequence of AUDs.

Scientific Significance—DNA methylation profiles may be used to assess AUD status or monitor AUD treatment response.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

INTRODUCTION

Alcohol use disorders (AUDs), including alcohol abuse and dependence, are prevalent and associated with a variety of medical and social problems. Family, twin, and adoption studies have shown that heritable factors play a critical role in determining an individual's vulnerability to AUDs^{1,2}; twin studies indicate that the heritability of AUDs is around 50–60%.^{3,4} The incomplete phenotypic concordance between monozygotic twins suggests that environmental and/or epigenetic factors are also important contributors to susceptibility to AUDs.

Although, AUD-associated genetic variants have been identified by genome-wide association studies (GWAS),^{5–10} they only explain a small proportion of the genetic variance for AUDs.^{11,12} While more variance will surely be explained when larger GWAS samples are collected or aggregated, the presently unexplained genetic variance may also be attributable in part to epigenetic events (such as DNA methylation, histone modifications, chromatin remodeling, and noncoding RNA regulations), rare or copy number variants, epistasis, or the interplay of genes and environment. Environmental factors alone (eg, chronic alcohol consumption) may also lead to alcohol tolerance or dependence through neuroadaptations. Increasing evidence suggests that drugs of abuse and drug-associated cues can alter gene expression in neurons, and this process may be mediated by epigenetic modifications (eg, DNA methylation).¹³ Thus, the development of AUDs over time may be influenced by neuroadaptations involving epigenetic modifications after long-term alcohol consumption.¹⁴

Epigenetic marks can be heritable during cell division¹⁵ but do not alter DNA sequence. Depending on the situation, they are potentially more important than genetic sequence variants in determining when, where, and how much a specific gene will be expressed during development. Epigenome-wide association studies (EWAS) have been applied to investigate the epigenetic mechanisms of diseases including AUDs. EWAS-identified epigenetic changes can be exploited clinically as biomarkers for disease diagnosis, prognosis, and treatment of AUDs and other diseases.

The best-studied kind of epigenetic modification of DNA in humans is methylation of cytosine at position C5 in CpG dinucleotides.¹⁶ More than 28 million CpG sites are distributed across the human genome,¹⁷ and 70–80% of them can be methylated.¹⁸ It is estimated that there are about 45,000 CpG islands (or clusters of CpG dinucleotides in GC-rich regions) per haploid human genome.¹⁹ CpG islands typically occur at or near the transcription start site (or the promoter region) of genes and are often hypomethylated.^{20,21} In the cell, methylation of CpG sites (especially those in CpG islands located in promoter regions) may influence gene transcription via at least two mechanisms: (i) DNA methylation inhibits the binding of transcription factors (TFs) to the promoter region, and thus, directly interferes with gene transcription^{22–24}; and (ii) DNA methylation attracts methyl CpG binding proteins (such as MeCP2) to the promoter region, and subsequently, methyl CpG binding proteins recruit a variety of histone deacetylase (HDAC) complexes and chromatin remodeling factors, leading to chromatin compaction and transcriptional repression.²⁵ DNA methylation at CpG dinucleotides plays a central role in many biological processes, such as

chromatin structure remodeling,²⁶ genomic imprinting,²⁷ transposon silencing,²⁸ chromosome X inactivation,²⁹ and cell differentiation.³⁰ DNA methylation can vary dynamically in response to developmental cues or environmental stimuli.³¹ There is also dynamic DNA methylation across different human cells and tissues.³² Altered DNA methylation may be involved in many psychiatric conditions, including AUDs.^{33–36}

Although, DNA methylation at the promoter regions of genes usually suppresses gene expression, the function of intragenic (within the gene body) or intergenic DNA methylation remains less clear. Either intragenic or intergenic DNA methylation may play an important role in gene expression regulation. The majority of methylated CpG islands are located in intragenic or intergenic regions, whereas less than 3% of CpG islands in the promoter region of genes were methylated.²⁴ Hypermethylation of intragenic regions (or gene bodies) may have at least two roles: (i) intragenic methylation prevents aberrant transcription from intragenic promoters; and (ii) intragenic methylation increases accuracy of splicing. Similarly, hypermethylation of intergenic regions can inhibit random (or nonspecific) gene transcription. To explore the influence of DNA methylation in regulatory, intragenic, or intergenic regions on the risk of AUDs, several studies have investigated AUD-associated DNA methylation alterations in the peripheral blood^{37–39} or postmortem brains^{40,41} of AUD subjects using microarray-based approaches.

Interaction of genetic variants and DNA methylation at CpG sites can influence an individual's vulnerability to disease. Single nucleotide polymorphisms (SNPs) can affect gene expression or function via mechanisms that are ultimately epigenetic by introducing or removing CpG dinucleotides (that then may or may not be sites of methylation). These are called CpG-SNPs. The two different parentally derived alleles can exhibit different methylation patterns; this is called allele-specific DNA methylation (ASM).^{42,43} A genome-wide survey showed that a significant proportion (38–88%) of ASM regions are dependent on the presence of heterozygous SNPs in CpG dinucleotides that can either lead to or disrupt their methylation potential.⁴⁴ SNPs can also influence DNA methylation across extended genomic regions. These SNPs are referred to as methylation quantitative trait loci (mQTLs). The strength of the association between SNPs and CpGs tends to decrease as the distance between them increases.⁴⁵ Two recent studies identified AUD-associated CpG-SNPs⁴⁶ or mQTLs.⁴⁵

Although, DNA methylation is an important regulator of gene transcription and altered DNA methylation may affect risk for many diseases, DNA methylation studies on AUDs are still at an early stage. Most DNA methylation studies on AUDs were published in the past 5 years. Compared to epigenetic studies on non-psychiatric diseases (eg, cancer), it is more challenging to study the epigenetic mechanisms of AUDs. This is because (i) AUDs are psychiatric conditions which have behavioral components plus a pharmacogenetic component and the direct pharmacological effects of alcohol. When comparing DNA methylome differences between AUD cases and controls, it is therefore, unknown whether epigenetic differences were already present before alcohol exposure (ie, are risk factors for AUDs) or whether they are consequent to chronic alcohol use; (ii) AUDs are highly comorbid with other substance use disorders and psychiatric illnesses, and thus, it is difficult to obtain a homogeneous sample (where subjects are diagnosed only with AUDs and there is

no comorbidity) for AUD study; and perhaps most importantly; (iii) for epigenetic studies, we would prefer to study the most relevant tissue. This can be easy for investigations of cancer epigenetics as the tumor tissue of interest is likely to be accessible. To understand the epigenetics of AUDs, we would prefer to study brain tissue. However, brain tissue samples from live AUD subjects are usually inaccessible. In spite of these difficulties, epigenetic studies (particularly DNA methylation studies) on AUDs are advancing quickly making use of those tissues that are available—blood and saliva from living subjects, and postmortem brain from deceased subjects. This review highlights the major findings on the association of DNA methylation and AUDs in human subjects as well as the most important and interesting future directions the research field may take in the near future.

METHODS

We conducted a systematic literature search of NCBI PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>) using keywords “DNA methylation” and “alcohol use disorders” or “alcohol dependence” or “alcohol abuse.” We included studies published prior to April 2016 in this review. Resulting abstracts were reviewed and included for discussion if they reported the association of DNA methylation and AUDs. Reference articles cited by these publications were also reviewed to detect additional studies that might have been missed in our literature search. Specifically, this review summarizes (i) AUD-associated global DNA methylation changes in repetitive DNA sequences; (ii) AUD-associated DNA methylation changes in promoter regions of candidate genes; (iii) AUD-associated DNA methylation changes across the genome; (iv) DNA methylation-gene expression correlations in AUD subjects; (v) the interactive effects of SNP genotypes and CpG methylation on AUDs; and (vi) novel approaches (about sampling and DNA methylation detection methods) for studying the relationship between DNA methylation and AUD risk.

RESULTS

AUD-Associated Global DNA Methylation Changes in Repetitive DNA Sequences

Global DNA methylation levels in repetitive DNA sequences can be detected by restriction digestion using methylation-sensitive endonucleases (eg, MspI cuts 5'-CCGG-3' independently of the methylation status of the sequence, while cutting of 5'-CCGG-3' by HpaII depends on the methylation status of the sequence).⁴⁷ Considering the high abundance of DNA repetitive elements such as the long interspersed nuclear element 1 (LINE-1) and the Alu sequence in the genome,⁴⁸ levels of global DNA methylation can also be estimated by the methylation status of DNA repetitive elements.⁴⁹ We identified six studies that examined AUD-or alcohol consumption-associated global DNA methylation changes in repetitive DNA sequences (Table 1).

Two published studies examined the potentiality of peripheral blood global DNA methylation as a biomarker of AUDs. Bonsch et al.⁵⁰ analyzed the relationship among global DNA methylation (determined by restriction digestion using methylation-sensitive endonucleases), plasma homocysteine, and AUDs in a case-control sample (90 AUD cases and 89 healthy controls). They observed a significant increase (by 10%) of global DNA methylation in AUD subjects who had elevated levels of homocysteine. Kim et al.⁵¹

evaluated methylation levels of repetitive element Alu in peripheral blood DNAs of 135 AUD patients and 150 healthy control subjects. They reported significantly higher methylation levels of Alu in AUD patients compared to healthy controls.

Alcohol consumption-induced global DNA methylation changes were examined in healthy individuals by Zhu et al.,⁴⁸ who examined the impact of alcohol drinking on Alu or LINE-1 methylation (quantified by PCR-pyrosequencing) in blood DNAs of 1,465 healthy subjects. They found an inverse correlation between alcohol drinking with methylation of Alu (but not LINE-1). In contrast, two other studies, one with 161 healthy control subjects⁵² and another with 384 healthy women,⁵³ failed to identify a significant effect of alcohol drinking on global methylation of blood DNAs. Additionally, Manzardo et al.⁴⁰ examined global DNA methylation patterns in postmortem frontal cortex tissues of 10 AUD patients and 10 matched controls using methylation DNA immunoprecipitation (MeDIP) and microarray assays, but no significant global DNA methylation differences between AUD patients and control subjects were observed.

Overall, the above studies, although not wholly consistent, suggest that global DNA methylation changes might play a complex role in the etiology of AUDs. To understand the mechanisms underlying this relationship, we need to uncover AUD-associated DNA methylation patterns in specific genes implicated in drug or alcohol dependence.

Candidate Gene Promoter DNA Methylation Changes in the Peripheral Blood of AUD Subjects

AUD-associated blood DNA methylation changes around transcription start sites or promoter regions of 12 candidate genes (*ANP*, *AVP*, *GDAPI*, *GRIN2B*, *HERP*, *MAOA*, *OPRM1*, *Orexin A*, *POMC*, *SLC6A3*, *SLC6A4*, and *SNCA*) have been examined in 14 published studies (Table 2). These genes exert effects on a variety of cellular functions, such as thirst and sodium balance (*AVP* [coding for arginine vasopressin] and *ANP* [coding for atrial natriuretic peptide]),⁵⁴ signal transduction during neuronal development (*GDAPI*, which encodes ganglioside induced differentiation associated protein 1), neurotransmission (*GRIN2B* [coding for NMDA receptor subtype 2B], *OPRM1* [coding for μ -opioid receptor], *POMC* [coding for proopiomelanocortin], *SLC6A3* [coding for dopamine transporter], and *SLC6A4* [coding for serotonin transporter]), endoplasmic reticulum (ER) stress response (*HERP*, which encodes homocysteine-induced endoplasmic reticulum protein),⁵⁵ monoamine oxidization (*MAOA*, which encodes monoamine oxidase A), sleep and arousal regulation (*Orexin A*, which encodes hypothalamic neuropeptide orexin A),⁵⁶ or presynaptic signaling and membrane trafficking (*SNCA*, which encodes synuclein, alpha).⁵⁷ Hillemacher et al.⁵⁸ compared *AVP* and *ANP* promoter DNA methylation differences between 111 AUD cases and 57 healthy controls. They reported a significant increase in *AVP* promoter DNA methylation but a significant decrease in *ANP* promoter DNA methylation in the peripheral blood of AUD subjects. A study by Bruckmann et al. with 49 AUD cases and 37 controls showed hypomethylation of *GDAPI* in AUD subjects.⁵⁹ Three of the above 12 genes showed hypermethylation in their promoter regions in the peripheral blood of AUD subjects: *HERP* (66 AUD cases/55 controls),⁶⁰ *OPRM1* (125 AUD cases/69 controls),⁶¹ and *SNCA* (84 AUD cases/93 controls).⁶² Four studies have examined *SLC6A3*

promoter DNA methylation changes in AUD subjects: two showed hypermethylation of *SCL6A3* in AUD subjects or significant associations of *SCL6A3* methylation and craving or alcohol cue-induced brain reactivity ([76 AUD cases/35 controls]⁶³; [38 AUD cases/17 controls]⁶⁴), while two others did not reveal significant differences in *SCL6A3* promoter DNA methylation levels between cases and controls ([100 AUD cases/98 controls]⁶⁵; [171 AUD cases/160 controls]⁶⁶). Additionally, the association of AUDs and DNA methylation in promoter regions of five other candidate genes (*GRIN2B*, *HCRT*, *SLC6A4*, *MAOA*, and *POMC*) were also examined, but the findings were either inconsistent or negative. Although, no significant DNA methylation alterations in promoter regions of *GRIN2B* (32 male AUD cases),⁶⁷ *MAOA* (191 adoptees),⁶⁸ and *POMC* (145 AUD cases/37 controls)⁶⁹ were observed in AUD subjects, DNA methylation levels in promoter regions of these three genes were significantly correlated with AUD symptoms (eg, craving or symptom counts). Additionally, negative findings were obtained from *Orexin A* (68 AUD cases)⁷⁰ and *SLC6A4* (192 adoptees).⁷¹ We note that most of the studies quoted above (especially the positive ones) have small sample size, and more work is clearly needed.

Considering that AUDs are complex disorders affected by multiple genes and gene–environment interactions, we may be certain that the above candidate gene studies missed a number of important susceptibility genes for AUDs. To circumvent this issue, we examined peripheral blood DNA methylation levels of 384 CpGs in promoter regions of 82 addiction-related genes in 285 African Americans (AAs; 141 AUD cases and 144 controls) and 249 European Americans (EAs; 144 AUD cases and 105 controls) using a custom-designed Illumina GoldenGate DNA methylation array. In African American AUD subjects, two promoter CpGs in two genes (the γ -aminobutyric acid receptor subunit beta 3 gene [*GABRB3*] and *POMC*) were hypermethylated; while in European American AUD subjects, six promoter CpGs in six genes (the serotonin receptor 3A gene [*HTR3A*], the neural cell adhesion molecule 1 gene [*NCAM1*], the dopamine receptor D4 gene [*DRD4*], the methyl-CpG binding domain protein 3 gene [*MBD3*], the serotonin receptor 2B gene [*HTR2B*], and the NMDA receptor subtype 1 gene [*GRIN1*]) were hypermethylated. A specific CpG site in the *HTR3A* promoter region showed a significantly higher methylation level in EA cases than in EA controls after Bonferroni correction. However, this extended candidate gene study is limited by several factors: (i) the density of promoter CpGs was not high, and thus, some functionally important promoter CpGs might have been ignored; (ii) gene body CpGs were not included; and (iii) novel genes for AUD susceptibility were not considered, recapitulating the fundamental problem of candidate gene studies.

AUD-Associated DNA Methylation Changes Across the Genome

Similar to GWAS, EWAS are non-hypothesis-driven study approaches. Recently, the EWAS approach was widely applied to study genome-wide DNA methylation alterations associated with a number of diseases including AUDs (Table 3). Two types of DNA methylation microarray platforms were commonly used: one was the Illumina Infinium HumanMethylation27 BeadChip, which covers 27,578 CpG sites located in the proximal promoter regions of 14,475 annotated genes (including 110 microRNA genes), and another was the Illumina Infinium HumanMethylation450 BeadChip, which covers 487,557 CpGs

across the genome and includes 94% of the Illumina Infinium HumanMethylation27 BeadChip probes.

Two published studies applied the Illumina Infinium HumanMethylation27 BeadChip assay to identify genes that were differentially methylated in AUD subjects.^{72,73} One study compared genome-wide blood DNA methylation differences between 63 male AUD patients and 65 male controls, and revealed differential methylation in a number of genes (including the alcohol dehydrogenases 1A gene [*ADH1A*], the alcohol dehydrogenases 7 gene [*ADH7*], the aldehyde dehydrogenase 3B2 gene [*ALDH3B2*], and the cytochrome P450 2A13 gene [*CYP2A13*]) that are involved in stress, immune response, signal transduction, and metabolism.⁷² Another study used the same DNA methylation platform to examine DNA methylomic profiles in the saliva of 309 hazardous drinkers, and identified multiple CpGs sites (including those sites near the aldehyde dehydrogenase 1 family, member A2 gene [*ALDH1A2*]) that were associated with loss of control over drinking.⁷³

Philibert et al. performed two EWAS studies on AUDs using the Illumina Infinium HumanMethylation450 BeadChip.^{37,39} Initially, they examined the impact of recent alcohol use on DNA methylomes using lymphoblast DNAs derived from 165 female adoptees, and found widespread changes in DNA methylation across the genome due to recent alcohol consumption.³⁷ In a follow-up study with 33 AUD patients and 33 controls, they also observed widespread DNA methylation changes in the peripheral blood of AUD subjects, and these changes were mapped to gene networks involved in apoptosis.³⁹ The most significant findings were generated from CpG sites in the promoter region of *GDAP1*, and the hypomethylation of *GDAP1* in alcohol dependent patients was validated by a recent study.⁵⁹ However, no gene was found to be differentially methylated at the genome-wide level when comparing AUD patients' methylomes at the beginning of treatment and after 4 weeks of treatment. Additionally, Zhao et al. compared peripheral blood DNA methylome patterns between 10 AUD subjects and 10 paired siblings (without AUDs).³⁸ They identified 865 hypomethylated and 716 hypermethylated CpG sites in peripheral blood DNAs of AUD subjects, and the most hypomethylated CpG site was located in the promoter of *SSTR4* (coding for somatostatin receptor 4) while the most hypermethylated CpG site was located in the promoter *GABRP* (coding for γ -aminobutyric acid A receptor, Pi).

DNA Methylation-Gene Expression Correlations in AUD Subjects

DNA methylation, as a common epigenetic mark in the genome, plays a critical role in the regulation of gene expression. Studies using cell lines, animal models, and human subjects have demonstrated that alcohol exposure (or consumption) results in DNA methylation changes, leading to altered gene expression. Khalid et al.⁷⁴ used human embryonic stem cells (hESCs) as a model to reveal the correlation of DNA methylation and gene expression in alcohol-treated hESCs. By integrative analysis of the transcriptomic and DNA methylomic data, they identified a list of cell differentiation-related genes that were dysregulated by alcohol-induced DNA methylation changes.

We examined alcohol drinking-induced brain *Htr3a* DNA methylation and gene expression alterations using the drinking-in-the-dark (DID) CD1 mouse model, and reported reduced methylation levels of *Htr3a* promoter CpGs in the dorsomedial striatum (DMSTR) but

elevated expression levels of *Htr3a* in the DMSTR in alcohol-drinking mice in comparison to water-drinking mice. Moreover, four studies have investigated the relationship between promoter DNA methylation and gene expression of several candidate genes in the peripheral blood (or lymphoblast cell lines derived from blood lymphocytes) of AUD subjects. A negative correlation of promoter DNA methylation levels with gene expression levels was observed in four candidate genes including *HERP*,⁶⁰ *DNMT-3b*,⁷⁵ *SLC6A4*,⁷¹ and *ANP*.⁵⁸ Additionally, Taqi et al.⁴⁶ observed a positive correlation of the methylation level of a SNP-CpG (rs2235749; C > T) located in the 3' UTR of the prodynorphin gene (*PDYN*) with the expressed level of dynorphins in postmortem dorsolateral prefrontal cortex (dl-PFC) of AUD subjects, thus, supporting the notion that gene body methylation can enhance gene transcription. However, we did not observe a clear-cut relationship between DNA methylation and gene expression at the genome-wide level in postmortem prefrontal cortex (PFC) of 16 male AUD patients and 16 matched male controls.⁴¹ The complex DNA methylation-gene expression relationship may be due to at least two mechanisms: (i) the influence of genetic variants on DNA methylation and/or gene expression confounds the relationship between DNA methylation and gene expression; and (ii) the gene-gene interaction network that leads to a complex relationship between DNA methylation and gene expression.

The Interactive Effect of SNP Genotypes and CpG Methylation on AUDs

To investigate the mechanisms of how DNA methylation regulates gene transcription, we must first understand the various biological influences on DNA methylation. Demographic factors, such as sex,⁷⁶ age,⁷⁷ and ethnicity,^{52,78} are known to impact or correlate with DNA methylation. DNA methylation patterns can also be influenced by various environmental exposures, including pre- and post-natal nutrition,^{79–81} early life stress,^{82–84} psychosocial stress,^{85,86} and inflammation.⁸⁷ Additionally, genetic variants (or mQTLs) can dramatically alter methylation levels of nearby CpGs. mQTLs are widely dispersed throughout the genome across different tissues.^{43,88–91} Three studies have examined the effect of genetic variants on methylation of proximal CpGs in AUD subjects. Philibert et al.⁶⁸ investigated the association between genotypes of the variable number tandem repeat (VNTR) polymorphism in the promoter region of *MAOA* and the overall methylation level of the *MAOA* promoter region using lymphoblastoid cell lines derived from blood lymphocytes of 191 subjects at high risk for AUDs. They reported that women homozygous for the 3,3 allele of the VNTR polymorphism had a trend ($p < 0.10$) of increased overall methylation of the *MAOA* promoter region than women homozygous for the 4,4 allele of the VNTR polymorphism. Taqi et al.⁴⁶ found that three AUD-associated *PDYN* CpG-SNPs, that is, SNPs that overlap with CpG sites, could directly alter the methylation status of these *PDYN* CpGs in postmortem dorsolateral prefrontal cortex (dl-PFC) of 14 AUD patients and 14 control subjects. In a recent study, we investigated genome-wide mQTLs that were associated with 384 CpGs in promoter regions of 82 addiction-related genes using peripheral blood DNAs of 268 African Americans (AAs: 129 AUD cases and 139 controls) and 143 European Americans (EAs: 129 AUD cases and 14 controls).⁴⁵ We identified 282 significant mQTL-CpG pairs in AAs and 313 significant mQTL-CpG pairs in EAs. These findings suggest that genetic variants (particular noncoding variants) may influence gene transcription via regulation of CpG methylation levels.

Overall, some of the above studies have demonstrated significant associations between DNA methylation and AUDs or AUD-related phenotypes, although inconsistent results have also been reported. These studies have significant limitations: (i) only a small proportion of CpG sites in the genome have been examined for their associations with AUDs (eg, the Illumina Infinium HumanMethylation450 BeadChip probes can only cover about 1.7% of CpGs in the human genome); (ii) few studies have examined AUD-associated DNA methylation changes in reward-related brain regions; and (iii) most importantly, very few studies have explored whether AUD-associated DNA methylation changes are inherited or resulted from chronic alcohol drinking. These issues are addressed below.

DISCUSSION

As mentioned earlier, epigenetic variation (such as DNA methylation) may be an explanation for some of the “missing heritability” of AUDs, considering that AUD-associated genetic variants identified by GWAS only explained a minority of the expected heritable fraction of AUDs.⁹² The findings from published studies suggest that DNA methylomic studies, when integrated with GWAS research, could uncover more of the genetic and epigenetic mechanisms of AUDs. Nevertheless, several methodological and scientific challenges in DNA methylomic studies must be addressed before fruitful results can be obtained.

Application of Novel DNA Methylome Profiling Technologies in Epigenetic Studies of AUDs

A number of studies have analyzed AUD-associated DNA methylation changes using DNA methylation analysis approaches that vary in genomic coverage and resolution. Global DNA methylation assays, based on examination of DNA methylation status of repetitive elements (such as Alu and LINE-1), are less expensive but DNA methylation alterations in specific genes or regions are unknown. Regional DNA methylation assays, which require several steps including bisulfite treatment, PCR amplification, and sequencing, are often time consuming and less efficient if multiple regions of interests (often promoter regions of genes) need to be investigated. Genome-wide DNA methylation assays, primarily based (so far) on two types of DNA methylation microarrays (27 and 450K) from Illumina, just probe a small fraction of CpGs in the human genome. To address these issues, Illumina has released an improved DNA methylation array, that is, the Infinium MethylationEPIC BeadChip, which covers more than 850K CpGs across the genome. Additionally, whole genome bisulfite sequencing (WGBS) (or bisulfite treatment of DNA followed by high-throughput sequencing) has been shown to be an important method for studying DNA methylation at single-base resolution,⁹³ and it has been applied to study epigenetic regulation mechanisms of diseases such as cancer.⁹⁴ However, WGBS is costly and genetic or epigenetic studies of psychiatric disorders (such as AUDs) usually require analyzing the data from a large sample (considering that genetic and epigenetic marks may exert a minor or moderate effect on psychiatric disorders). With the decline of the DNA sequencing cost, it is expected that WGBS should be applied more widely in DNA methylomic studies of AUDs or other complex disorders, if appropriate funding becomes available. For example, the Reduced Representation Bisulfite Sequencing (RRBS) method developed by Diagenode

(www.diagenode.com) is a cost-effective and high throughput approach, and it provides improved coverage of up to four million CpGs for the human genome.

Investigation of AUD-Associated DNA Methylation Alterations in Reward-Related Brain Region

To enhance our understanding of the etiology of AUDs, we need to examine AUD-associated DNA methylation alterations in reward-related brain regions. Although, blood or saliva samples are easily accessible and blood or saliva DNA methylation changes can be valuable biomarkers for diseases including AUDs, the extent to which DNA methylation in the peripheral blood or saliva reflects that in the brain is not well established.⁹⁵ Given that the reinforcing effects of alcohol are mediated through reward-related brain regions (eg, the ventral tegmental area, the nucleus accumbens, the prefrontal cortex, etc.), it would be desirable to investigate DNA methylation changes in these brain regions of AUD subjects. Another possibility would be to investigate the epigenetics of these brain regions in animal models. In our recent study using DID mice as models discussed above, we examined alcohol-induced DNA methylation changes in the promoter region of *Htr3a* in nine reward-related brain regions of mice and showed a wide range of alterations in the methylation of this gene in different brain regions.⁹⁶ These results indicate that DNA methylation can be tissue-specific. A small human subject study using blood and brain tissue samples from two men and one woman revealed that tissue-specific epigenetic variation outweighed between-individual epigenetic variation.⁹⁷ Therefore, the identification of DNA methylation changes in different brain regions of AUD subjects is an important aim of our future studies in order to have a better understanding of the particular function of specific brain regions involved in AUD development.

To date, only three small published studies have analyzed DNA methylation changes in postmortem brains of AUD subjects (Table 4). Taqi et al.⁴⁶ examined methylation levels of CpGs in the *PDYN* in postmortem PFC of 14 AUD patients and 14 control subjects. They reported altered methylation of three SNP-CpGs in postmortem PFC of AUD subjects. Another study examined global methylation alterations in postmortem frontal cortex of AUD subjects, but no significant findings were obtained.⁴⁰ The most recent study from our lab examined DNA methylation alterations in postmortem PFC of 16 pairs of male and 7 pairs of female AUD and control subjects (46 subjects in total).⁴¹ We identified 1,812 CpGs (in 1,099 genes) that were differentially methylated in male AUD subjects. The genes carrying these differentially methylated CpGs are involved preferentially in neural development and transcriptional regulation. Although, these postmortem brain epigenetic studies have generated interesting findings, they are limited by the small sizes of brain tissue samples and the unavailability of tissues from multiple reward-related brain regions. These limitations will be overcome with the increased access of a larger number of human brain tissues from human brain banks, such as the New South Wales Tissue Resource Centre (NSW-TRC) at the University of Sydney (Australia)⁹⁸ that collects postmortem brain tissues from schizophrenia or AUD patients and matched healthy control subjects.

Unraveling the Mechanisms of Differential DNA Methylation in AUD Subjects

As noted in our review of the literature, a number of human studies have supported altered DNA methylation in either the peripheral blood or postmortem brains of AUD subjects. However, it is unclear whether DNA methylation changes in AUD subjects result directly from alcohol consumption or predate the occurrence of alcohol abuse or dependence, and whether altered DNA methylation influences gene expression. It is already known that environmental factors such as early life stress and malnutrition (that may be abnormal in AUD subjects) can also alter DNA methylation patterns.⁹⁹ Moreover, DNA methylation status in AUD subjects can be influenced by alcohol doses as well as co-occurring smoking, drug use, or comorbid psychiatric illness. To unravel the mechanisms underlying DNA methylation alterations observed in AUD subjects, at least four study approaches can be used.

The first approach is to use animal models to validate DNA methylation/gene expression alterations observed in AUD subjects. For instance, following the finding that a CpG site in the promoter region of *HTR3A* was hypermethylated in the peripheral blood of AUD subjects,¹⁰⁰ our research group used the DID mouse model to validate the finding from human AUD subjects.⁹⁶ We also observed increased methylation levels of *Htr3a* promoter CpGs in the blood of mice exposed to alcohol. Moreover, we observed altered methylation levels of *Htr3a* promoter CpGs in reward-related brain regions (elevated in the hippocampus but reduced in the dorsomedial striatum and the dorsomedial prefrontal cortex) of alcohol drinking mice. Additionally, we found that *Htr3a* promoter methylation levels were inversely correlated with *Htr3a* expression levels in specific brain regions such as the dorsomedial striatum. Nevertheless, particular caution must be taken when extrapolating from the mouse findings because the promoter DNA sequence of human *HTR3A* and mouse *Htr3a* are not homologous.

The second approach is to use stem cell-derived neurons to model DNA methylation/gene expression alterations in the brain neurons of AUD subjects. It has been hypothesized that chronic alcohol consumption might result in sustained epigenetic and gene expression alterations in brain neurons, leading to neuroadaptations to alcohol. This hypothesis cannot be tested directly in live human brains. Recent advances in stem cell biology provide a novel opportunity for recapitulating the genetic and epigenetic mechanisms underlying diseases such as AUDs in a culture dish. Excitatory neurons (eg, glutamatergic neurons) and inhibitory neurons (eg, GABAergic neurons), which play a critical role in drug addiction, can be differentiated from human embryonic stem cells (hESCs).^{101–103} Moreover, stem cell-derived neurons have been demonstrated to be useful in in vitro cellular models to evaluate the function of genetic variants associated with neuropsychiatric disorders (eg, schizophrenia¹⁰⁴) and neurodevelopmental disorders (eg, the Rett Syndrome¹⁰⁵). With the use of stem cell-derived excitatory or inhibitory neurons as cellular models, we expect to obtain critical information on alcohol consumption-induced epigenomic and transcriptomic changes, thus, mimicking those changes in the brain neurons of AUD subjects.

The third approach, quite different in its goals from the methods described above, is to perform longitudinal or multi-generation epigenetic studies to confirm whether AUD-associated DNA methylation changes are attributable to alcohol consumption, environmental

factors, or inherited from parents, or as seems most likely, some combination of these. Longitudinal studies have distinct advantages over cross-section studies (which were applied in most published studies on epigenetics of AUDs). Importantly, longitudinal studies do not need to consider the influence of genetic variation on DNA methylation, and thus, can directly identify the temporal or dynamic DNA methylation changes in individual subjects. It is expected that a longitudinal survey can provide direct evidence that AUD-associated DNA methylation changes are a result of alcohol consumption. To date, only two studies are known to have used the longitudinal DNA methylation study approach to identify DNA methylation changes associated with chronic alcohol consumption. One study analyzed *AVP* and *ANP* promoter DNA methylation changes in the peripheral blood of 99 AUD patients on days 1, 7, and 14 of alcohol withdrawal,¹⁰⁶ and the another study tracked blood DNA methylomic changes in 10 AUD patients (normal in phases 1 and 2 but affected with AUDs in phase 3) and 10 controls (unaffected with AUDs) in three phases (phase 1: 1986; phase 2: 1990–1992; and phase 3: 2003–2009).¹⁰⁷ These two longitudinal studies support that alcohol consumption influences DNA methylation, and genome-wide DNA methylation drifts over time. Additionally, multigenerational epigenetic studies could elucidate whether AUD-associated DNA methylation changes are more likely to be inherited from parents. Studies have shown that not only maternal smoking but also grandmaternal smoking is associated with elevated pediatric asthma risk in offspring.¹⁰⁸ Using a rat model, it has been demonstrated that in utero nicotine exposure results in alterations to both the somatic and germ cell epigenome.¹⁰⁹ Thus, epigenetic mechanisms appear to underlie some part of the multigenerational transmission of a nicotine-induced asthma-like phenotype. Since many women continue to smoke and/or drink during pregnancy, the negative impact of tobacco or alcohol on the epigenome of the fetus can begin before birth and can influence the vulnerability of children to nicotine or alcohol addiction in their adult life. No published studies have used the multigenerational study approach to investigate the epigenetic mechanisms of AUDs.

The fourth approach is to identify AUD-specific DNA methylation changes using a well-characterized and well-powered sample of AUD cases, and matched healthy controls. DNA methylation status is affected by the frequency or severity of alcohol use.³⁷ Instead of relying on self-report measures of alcohol use, ascertaining alcohol use status by treatment setting (ie, inpatient detoxification) or measuring blood alcohol levels for characterizing AUD status may be advantageous for examination of AUD-associated DNA methylation alterations. However, this also carries the substantial limitation of dosage ascertainment, and only a limited number of inspections are conducted over a lifetime or even across generations, while epigenetic marks are more likely to develop over the long term. Additionally, AUD subjects often have comorbid smoking, drug use, or psychiatric disorders (such as schizophrenia, bipolar disorders, and depression).¹¹⁰ It is necessary to clarify whether DNA methylation changes observed in AUD subjects are attributable to alcohol consumption or due to comorbid nicotine or drug use, or other psychiatric disorders. When analyzing AUD-associated DNA methylation changes, we can exclude those subjects who use illicit drugs of abuse or are affected with psychiatric disorders, or alternatively, adjust analytically for comorbid phenotypes. Nevertheless, if we exclude those subjects who are also smokers, the sample size for the study will be significantly reduced; individuals with

AUDs are four times more likely to be smokers than those without AUDs, and nearly 23% of smokers met past-year criteria for AUDs.^{111–113} To identify AUD-specific DNA methylation changes, we need to use a sample of AUD case and control subjects who are matched in smoking status and consider the status of smoking as a confounding factor in the data analysis.

Finally, when analyzing DNA methylation changes in the peripheral blood of AUD subjects, we need to consider the proportion of different types of blood cells of each subject. Numerous studies have examined DNA methylation alterations in peripheral blood mononuclear cells (PBMCs, including T, NK, and B cells as well as monocytes) of patients with various diseases such as alcohol dependence.³⁸ However, variations in DNA methylation profiles among different populations of PBMCs have also been reported.¹¹⁴ To examine AUD-associated blood DNA methylation changes, the proportions of different types of blood cells (eg, CD8T, CD4T, NK cells, B cells, monocytes, granulocytes, etc.) must be taken into consideration, as described in published studies.^{114,115}

CONCLUSION

The work reviewed herein provides evidence that DNA methylation may play an important role in AUDs via regulation of gene transcription. Even though there are many challenges in the study of the epigenetic mechanisms of AUDs, the advances of new technologies for DNA methylomic profiling, the availability of postmortem brain tissues, stem cell technology could improve our knowledge regarding the cause and function of DNA methylation changes in the risk for AUDs. We also look forward to the development of novel approaches that can provide new ways to address the methodological limitations discussed in this article.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) grants R21 AA023068 (HZ), R01 AA025080 (HZ), R01 AA017535 (JG), R01 DA012690 (JG), and P50 AA12870 (JG). The reviewers' comments were very helpful for improving the manuscript.

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TABLE 1

Alcohol use disorder or alcohol consumption-associated global DNA methylation changes

Sample	Diagnosis	Tissue	Global DNA methylation assay	Results
Bonsch et al. ⁵⁰ 90 male AUD cases/89 controls	DSM-IV	Peripheral blood	Endonuclease digestion	Elevated methylation in cases ($p = .002$)
Kim et al. ⁵¹ 135 male AUD cases/150 controls	DSM-V	Peripheral blood	Pyrosequencing of Alu	Elevated methylation in cases ($p < .0001$)
Zhu et al. ⁴⁸ 1,465 healthy subjects	N/A	Peripheral blood	Pyrosequencing of Alu and LINE-1	Inverse correlation between alcohol drinking with methylation of Alu (but not LINE-1) ($\beta = -.214$)
Zhang et al. ⁵² 161 healthy subjects	N/A	Peripheral blood	Pyrosequencing of LINE-1	No effect of alcohol drinking on methylation
Ono et al. ⁵³ 384 healthy women	N/A	Peripheral blood	Endonuclease digestion	No effect of alcohol drinking on methylation
Manzardo et al. ⁴⁰ 10 AUD cases/10 controls	DSM-IV	Postmortem frontal cortex	Methylated DNA immunoprecipitation plus microarray assays	No significant differences in methylation between cases and control

DSM IV/V, The Diagnostic and Statistical Manual of Mental Disorders, 4th/5th edition. N/A, Not available; AUD, Alcohol use disorder.

TABLE 2
Alcohol use disorder or alcohol consumption-associated DNA methylation changes in candidate genes

Sample	Diagnosis	Genes	Methylation assay	Results
Hillemecher et al. ⁵⁸				
111 AUD cases/57 controls	DSM-IV/ICD-10	<i>AVP/ANP</i>	Real-time PCR	AVP hypermethylation in cases ($p=.029$) ANP hypomethylation in cases ($p=.023$)
Bruckmann et al. ⁵⁹				
49 AUD cases/37 controls	DSM-IV	<i>GDAPI</i>	Pyrosequencing	Hypomethylation in cases ($p=.001$)
Biermann et al. ⁶⁷				
32 male AUD cases	DSM-IV	<i>GRIN2B</i>	Bisulfite sequencing	Drinking severity was negatively correlated with methylation ($p=.013$)
Bleich et al. ⁶⁰				
66 male AUD cases/55 controls	DSM-IV	<i>HERP</i>	Endonuclease digestion/PCR	Hypermethylation in cases ($p<.020$)
Philibert et al. ⁶⁸				
191 adoptees	SSAGA-II/DSM-IV	<i>MAOA</i>	Mass spectrometry	Methylation associated with AUD symptom counts in women ($p<.008$)
Zhang et al. ⁶¹				
125 AUD cases/69 controls	SSADDA	<i>OPRMI</i>	Bisulfite sequencing	Hypermethylation in cases ($p=.004-.033$)
Bayerlein et al. ⁷⁰				
68 AUD cases	DSM-IV/ICD-10	<i>Orexin A</i>	Bisulfite sequencing	No significant association with AUD symptoms
Muschler et al. ⁶⁹				
145 AUD cases/37 controls	OCDS	<i>POMC</i>	Bisulfite sequencing	No significant difference; Associated with alcohol craving ($p=.023$)
Hillemecher et al. ⁶³				
76 AUD cases/35 controls	DSM-IV/ICD-10	<i>SLC6A3</i>	Real-time PCR	Hypermethylation in cases ($p=.001$)
Wiers et al. ⁶⁴				
38 AUD cases/17 controls	DSM-IV	<i>SLC6A3</i>	Bisulfite sequencing	Hypermethylation in cases ($p=.008$)
Nieratschker et al. ⁶⁵				
100 AUD cases/98 controls	DSM-IV	<i>SLC6A3</i>	Bisulfite sequencing	No significant difference
Jasiewicz et al. ⁶⁶				
171 AUD cases/160 controls	ICD-10	<i>SLC6A3</i>	Methylation-specific PCR	No significant difference
Philibert et al. ⁷¹				

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Sample	Diagnosis	Genes	Methylation assay	Results
192 adoptees Bonsch et al. ⁶²	SSAGA-II/DSM-IV	<i>SLC6A4</i>	Mass spectrometry	No association between methylation and AUD history
84 AUD cases/93 controls Zhang et al. ¹⁰⁰	DSM-IV	<i>SNAC</i>	Endonuclease digestion/PCR	Hypermethylation in cases ($p = .021$)
141 AA AUD case/144 AA controls; 144 EA AUD case/105 EA controls	SSADDA	82 addiction-related genes	Illumina	Hypermethylation of GABRB3 and POMC in AA cases ($p < .001$); hypermethylation of HTR3A, NCAM1, DRD4, MBD3, HTR2B, and GRIN1 in EA cases ($p < .001$)
GoldenGate Methylation Array				

DSM-IV, The Diagnostic and Statistical Manual of Mental Disorders, 4th edition; ICD-10, The International Statistical Classification of Diseases and Related Health Problems, 10th Revision; SSAGA-II, The Semi-Structured Assessment for the Genetics of Alcoholism, version 2; SSADDA, The Semi-Structured Assessment for Drug Dependence and Alcoholism; OCDs, The Obsessive Compulsive Drinking Scale; AUD, Alcohol use disorder; AA, African American; EA, European American.

TABLE 3

Alcohol use disorder or alcohol consumption-associated blood or saliva DNA methylomic changes

Sample	Diagnosis	Tissue	Methylation assay	Results
Zhang et al. ⁷² 63 male AUD cases/65 controls	DSM-IV	Peripheral blood	Illumina Infinium HumanMethylation27 BeadChip	1,710 CpGs were differentially methylated; most of the top CpGs were hypomethylated in cases ($p < .005$)
Harlaar et al. ⁷³ 309 hazardous drinkers	ICS/AD/AUDIT	Saliva	Illumina Infinium HumanMethylation27 BeadChip	Significant associations between loss of control over drinking and DNA methylation at multiple CpG sites (eg, a CpG near the ALDHA2 gene)
Philibert et al. ³⁷ 165 female adoptees	SSAGA-II	Lymphoblastoid cell lines	Illumina Infinium HumanMethylation450 BeadChip	Alcohol consumption caused widespread DNA methylation changes across the genome
Zhao et al. ³⁸ 10 AUD cases/10 paired siblings	DSM-IV/AUDIT	Peripheral blood	Illumina Infinium HumanMethylation450 BeadChip	865 hypomethylated CpGs and 716 hypermethylated CGs in cases
Philibert et al. ³⁹ 33 AUD cases/33 controls	SSAGA-II	Peripheral blood	Illumina Infinium HumanMethylation450 BeadChip	Widespread DNA methylation changes in the genomes of cases

DSM IV, The Diagnostic and Statistical Manual of Mental Disorders, 4th edition; ICS, The Impaired Control Scale; ADS, The Alcohol Dependence Scale; AUDIT, The Alcohol Use Disorder Identification Test; SSAGA-II, The Semi-Structured Assessment for the Genetics of Alcoholism, version 2; AUD: Alcohol use disorder.

TABLE 4

Alcohol use disorder-associated DNA methylomics changes in postmortem brains

Sample	Diagnosis	Tissue	Methylation assay	Results
Taqi et al. ⁴⁶ 14 male AUD cases/14 controls	DSM-IV	Dorsolateral prefrontal cortex	Bisulfite sequencing of <i>PDYN</i>	Differential methylation of three <i>PDYN</i> SNP-CpGs
Manzardo et al. ⁴⁰ 10 AUD cases 10 controls	DSM-IV	Prefrontal cortex	DNA immunoprecipitation plus microarray assays	No significant differences in global DNA methylation between cases and control
Wang et al. ⁴¹ 16 male AUD cases/16 male controls; 7 female AUD cases/7 female controls	DSM-IV	Prefrontal cortex	Illumina Infinium HumanMethylatio n450 BeadChip	In male cases: 1,812 CpGs (in 1,099 genes) were differentially methylated ($9.5 \times 10^{-9} < p_{\text{nominal}} < 7.2 \times 10^{-4}$); In female cases: 154 CpGs (in 104 genes) were differentially methylated ($p_{\text{nominal}} < 1.0 \times 10^{-3}$)

DSM IV, The Diagnostic and Statistical Manual of Mental Disorders, 4th edition; AUD, Alcohol use disorder.