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Array-based Profiling of DNA Methylation Changes Associated with Alcohol Dependence

Huiping Zhang[1,6,*], **Aryeh I. Herman**[1,6], **Henry R. Kranzler**[7], **Raymond F. Anton**[8], **Hongyu Zhao**[2,5], **Wei Zheng**[3], and **Joel Gelernter**[1,2,4,6]

[1]Department of Psychiatry, Yale University School of Medicine, New Haven, CT

[2]Department of Genetics, Yale University School of Medicine, New Haven, CT

[3]Department of Molecular Biophysics & Biochemistry, Yale University School of Medicine, New Haven, CT

[4]Department of Neurobiology, Yale University School of Medicine, New Haven, CT

[5]Division of Biostatistics, Yale University School of Public Health, New Haven, CT

[6]VA Connecticut Healthcare System, West Haven, CT

[7]Department of Psychiatry, University of Pennsylvania Perelman School of Medicine and VISN4 MIRECC, Philadelphia VAMC, Philadelphia, PA

[8]Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina, Charleston, SC

Abstract

Background—Epigenetic regulation through DNA methylation may influence vulnerability to numerous disorders, including alcohol dependence (AD).

Methods—Peripheral blood DNA methylation levels of 384 CpGs in the promoter regions of 82 candidate genes were examined in 285 African Americans (AAs; 141 AD cases and 144 controls) and 249 European Americans (EAs; 144 AD cases and 105 controls) using Illumina GoldenGate Methylation Array assays. Association of AD and DNA methylation changes were analyzed using multivariate analyses of covariance with frequency of intoxication, sex, age and ancestry proportion as covariates. CpGs showing significant methylation alterations in AD cases were further examined in a replication sample (49 EA cases and 32 EA controls) using Sequenom's MassARRAY EpiTYPER technology.

[*]Correspondence to: Huiping Zhang, Ph.D., Department of Psychiatry, Yale University School of Medicine, VA Medical Center/ 116A2, 950 Campbell Avenue, West Haven, CT 06516, USA, Tel: (203) 932-5711 ext. 5245, Fax: (203) 937-4741, huiping.zhang@yale.edu.

FINANCIAL DISCLOSURES

Dr. Kranzler has received compensation for professional services from the National Institutes of Health (NIAAA and NIDA) and for academic lectures and editorial functions in various scientific venues (including the ACNP). Dr. Kranzler has had consulting arrangements with the following pharmaceutical companies: Alkermes, Gilead, GlaxoSmithKline, Lilly, Lundbeck, Pfizer and Roche. Dr. Anton has had consulting agreements with the following companies: Eli Lilly, GlaxoSmithKline, Alkermes, Lundbeck and Roche. Drs. Kranzler and Anton also receive support from the ACNP Alcohol Clinical Trials Initiative (ACTIVE), which Eli Lilly, Schering Plough, Lundbeck, Alkermes, GlaxoSmithKline, Abbott, and Johnson & Johnson support. Drs. Kranzler and Anton report research support from Merck and Dr. Anton from Eli Lilly. Dr. Gelernter reports that he has received compensation for professional services in the previous three years from the following entities: Yale University School of Medicine, Veterans Affairs Healthcare System (VA), and the National Institutes of Health (NIAAA, NIDA, and NIMH), and related to academic lectures and editorial functions in various scientific venues (including the ACNP). Other authors have no conflict of interest to report.

Results—In AAs, two CpGs in two genes (*GABRB3* and *POMC*) were hypermethylated in AD cases compared to controls ($P \leq 0.001$). In EAs, six CpGs in six genes (*HTR3A*, *NCAM1*, *DRD4*, *MBD3*, *HTR2B* and *GRIN1*) were hypermethylated in AD cases compared to controls ($P \leq 0.001$); CpG cg08989585 in the *HTR3A* promoter region showed a significantly higher methylation level in EA cases than in EA controls after Bonferroni correction ($P = 0.00007$). Additionally, methylation levels of six CpGs (including cg08989585) in the *HTR3A* promoter region were analyzed in the replication sample. Although the six *HTR3A* promoter CpGs did not show significant methylation differences between EA cases and EA controls ($P = 0.067–0.877$), the methylation level of CpG cg08989585 was non-significantly higher in EA cases (26.9%) than in EA controls (18.6%) ($P = 0.139$).

Conclusions—The findings from this study suggest that DNA methylation profile appears to be associated with AD in a population-specific way and the predisposition to AD may result from a complex interplay of genetic variation and epigenetic modifications.

Keywords

Illumna GoldenGate Methylation Array; Sequenom MassARRAY EpiTYPER; Promoter CpGs; Alcohol Dependence; Peripheral Blood DNA

INTRODUCTION

Alcohol dependence (AD) is a common psychiatric disorder with heterogeneous etiology. Family, twin and adoption studies show that heritable factors play a critical role in determining an individual's vulnerability to AD (Ball, 2008; Kohnke, 2008). Twin studies indicate that the heritability of AD is around 50–60% (Enoch and Goldman, 2001; Goldman et al., 2005). The incomplete phenotypic concordance between monozygotic twins suggests that environmental and epigenetic factors are important contributors to the susceptibility to AD. Hence, AD is now commonly viewed as a complex disorder that results from the interaction of genetic variation, environmental factors and epigenetic modifications. While a great deal of research has focused on genetic and environmental effects, and to a lesser extent on gene-environment interactions, few studies have examined the epigenetic profile in AD.

Epigenetic modifications, such as DNA methylation and histone modification, are regulatory mechanisms that alter gene expression without changing DNA sequence. Epigenetic changes may have a considerable impact on gene transcription and physiological and pathophysiological processes, leading to altered risk for diseases. Additionally, epigenetic modifications are essential for appropriate cellular development and differentiation in mammals (Franklin and Mansuy, 2010; Kramer, 2005). One of the best-studied epigenetic mechanisms is DNA methylation, which may change promoter activity and thus regulate gene transcription.

Accumulating data indicate that alcohol abuse can alter the methylation status of specific genes. An initial study by Bonsch *et al.*(2005) showed a higher peripheral blood DNA methylation level in the α-synuclein gene (*SNCA*) in AD patients than in controls. Subsequent studies using peripheral blood (or lymphoblastoid cell lines derived from peripheral blood lymphocytes) indicated that several other genes, including the monoamine oxidase A gene (*MAOA*) (Philibert et al., 2008a), the serotonin transporter gene (*SLC6A4*) (Philibert et al., 2008b), the N-methyl-D-aspartate receptor subunit 2B gene (*NR2B or GRIN2B*) (Biermann et al., 2009), and the proopiomelanocortin gene (*POMC*) (Muschler et al., 2010) were more highly methylated in subjects with AD than in controls. A recent study using postmortem specimens of human dorsolateral prefrontal cortex demonstrated increased methylation of a CpG-SNP (rs2235749; C > T) in the 3′ untranslated region (3′

UTR) of the prodynorphin gene (*PDYN*) (Taqi et al., 2011). Moreover, an inverse correlation of promoter DNA methylation levels and gene expression levels has been observed. For example, Bonsch *et al.* (2006) showed that genomic DNA hypermethylation was associated with lower mRNA levels of DNA methyltransferase gene (*DNMT3B*) in patients with AD. Similarly, hypermethylation of the promoter region of the homocysteine-induced endoplasmic reticulum protein gene (*HERP*) was associated with down-regulation of *HERP* expression in patients with AD (Bleich et al., 2006).

Published studies comparing methylation levels in AD cases and controls have been limited to only several candidate genes. Because AD is a complex disorder affected by multiple genes and gene-environment interactions, a number of important AD susceptibility genes are likely to have been ignored. To understand AD-associated DNA methylation alterations, multiple genes or genes in a specific pathway or even in the whole genome should be examined. In this study, we systematically examined DNA methylation levels for genes participating in several important biological pathways (e.g., brain neurotransmission, alcohol metabolism, and DNA methylation) in a case-control sample. This enabled us to identify CpG sites (or genes) with significantly different methylation levels in AD cases compared with controls.

MATERIALS AND METHODS

Participants

Two hundred eighty-five African Americans (AAs) (141 AD cases and 144 healthy controls) and 249 European Americans (EAs) (144 AD cases and 105 healthy controls) (Set I sample) were recruited from substance abuse treatment centers and through advertisements at the University of Connecticut Health Center (n=260), Yale University (n=139), and the Medical University of South Carolina (n=135). The replication sample (Set II sample) included 49 EA cases with AD and 32 EA controls (Table 1). Both cases and controls were chosen from a larger sample of subjects recruited for studies of the genetics of substance dependence. Information on co-occurring diagnoses for cocaine or opioid dependence (CD or OD), days of intoxication in the past 30 days, and years of intoxication lifetime is presented in Table 1. Subjects were interviewed using an electronic version of the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) (Pierucci-Lagha et al., 2005) and lifetime diagnoses for AD were made according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (American Psychiatric Association, 1994). Individuals with a lifetime major psychotic disorder (schizophrenia or bipolar disorder) were excluded from both case and control groups. Controls were screened to exclude those with alcohol or drug abuse or dependence. Subjects gave informed consent as approved by the institutional review board at each clinical site, and certificates of confidentiality were obtained from the National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism.

DNA Extraction and Bisulfite Modification

Genomic DNA was extracted from peripheral blood using the PAXgene Blood DNA Kit (PreAnalytiX, Hombrechtikon, Switzerland). One microgram of genomic DNA was treated with the bisulfite reagent included in the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). Unmethylated cytosines were converted to uracils while methylated cytosines remained unchanged (Wang et al., 1980). Bisulfite-converted DNA samples were then used in the Illumina GoldenGate Methylation assay.

Designing the Custom Illumina GoldenGate Methylation Profiling Panel

The Illumina GoldenGate Assay for Methylation is a customizable method to analyze the methylation status of 384 to 1,536 CpG sites simultaneously. To design a custom methylation panel, we prepared an input (or GeneList) file to query all CpGs in the region extending from 2,000 bp upstream to 1,000 bp downstream from the transcription start sites of 82 candidate genes. An output (or GGMAScore) file was generated by Illumina (Illumina, San Diego, CA, USA) using the Assay Design Tool. We edited the GGMAScore file to remove CpGs predicted to perform poorly using the following criteria: (1) a methylation assay score that failed to be ≥ 0.50 (mean±S.D.: 0.86±0.06); (2) no known polymorphisms within the probe region; and (3) no CpGs in the assay pool within 60 nucleotides of each other. Based on these criteria, 384 CpGs in the 82 candidate genes were selected. Finally, the Oligo Pool for Methylation Assay (OMA) of the selected 384 CpGs was manufactured by Illumina (Illumina, San Diego, CA, USA). The sequence information for the 384 designated CpG sites is included in Supplementary Table S1. The 82 candidate genes are involved in several brain neurotransmission systems (dopaminergic, opioidergic, serotonergic, GABAergic/glutamatergic, cholinergic, and cannabinoidergic), alcohol metabolism, DNA methylation, or signal transduction for alcohol reward and reinforcement (Figure 1). A majority of these genes were previously represented in a SNP genotyping array for genetic association studies of AD and other substance dependence disorders (Hodgkinson et al., 2008). Several other candidate genes were selected according to previous genetic association study results (e.g., *NCAM1*, *TTC12* and *ANKK1*) (Gelernter et al., 2006; Yang et al., 2007) or based on their function in DNA methylation (e.g., *DNMT1*, *DNMT3A*, *DNMT3B* and *MECP2*). One to 12 CpGs in each of the 82 candidate genes were included in the custom methylation profiling panel.

Illumina GoldenGate Methylation Array Assay

After bisulfite conversion of genomic DNA, the remaining methylation assay steps were similar to the GoldenGate genotyping assay (Fan et al., 2003), except that four oligonucleotides, two allele-specific oligonucleotides (ASOs) and two locus-specific oligonucleotides (LSOs), were required for each CpG site rather than the three oligonucleotides required for SNP genotyping (Bibikova et al., 2006). Image processing and intensity data extraction were performed using the Illumina GenomeStudio™ Methylation Module v.1.0 Software. The background normalization algorithm was used to minimize background variation within the array by using built-in negative control signals. The methylation level (defined as β) of each individual CpG site was estimated as the ratio of intensities between methylated and unmethylated alleles. The β value was calculated as: $\beta = [\text{Max}(Cy5,0)]/[\text{Max}(Cy3,0) + \text{Max}(Cy5,0) + 100]$. It ranges from 0 in the case of completely unmethylated sites to 1 in completely methylated sites. To monitor both bisulfite conversion efficiency and accuracy of methylation detection, internal controls and technical controls were included in the methylation assay. The internal controls consisted of a methylated human DNA standard and a non-methylated human DNA standard (Zymo Research, Orange, CA, USA). Additionally, 5% of the bisulfite-converted human DNA samples (534 × 5% = 27) were replicated in the DNA methylation assay, and these were considered as technical controls. CpG methylation assays were highly reproducible within arrays (r^2=0.995) and between arrays (r^2=0.992) (Supplementary Figure S1).

Analysis of AD-associated DNA Methylation Alterations

The effect of AD on DNA methylation was analyzed using the multivariate analysis of covariance (MANCOVA) with the number of days of intoxication in the past 30 days, the number of years of intoxication lifetime, sex, age, and ancestry proportions as covariates. To address the potential influence of comorbid CD or OD, we also compared DNA methylation differences between healthy controls and 1) AD only cases (with no comorbid CD or OD),

2) AD+CD cases (AD cases with comorbid CD), 3) AD+OD cases (AD cases with comorbid OD), and 4) AD+CD+OD cases (AD cases with comorbid CD and OD)]. The African or European ancestry proportion of each subject was estimated by examining a set of ancestry informative markers (AIMs) as described in our previous study (Zhang et al., 2009). Additionally, the effect of sex on DNA methylation was analyzed using MANCOVA and the correlation between age and DNA methylation was analyzed using the Pearson correlation coefficient in AA and EA healthy controls. All of these analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The significance of the *P* values obtained from the MANCOVA was adjusted by Bonferroni correction.

Verification of AD-associated DNA Methylation by Sequenom MassARRAY EpiTYPER

To confirm AD-associated DNA methylation changes (observed with the Illumina methylation array-based assay) in the *HTR3A* promoter region, we used the Sequenom MassARRAY EpiTYPER approach (Sequenom, San Diego, CA, USA) to analyze a set of replication samples (Set II: 49 EA cases and 32 EA controls, see Table 1). Briefly, two *HTR3A* promoter amplicons [harboring eight CpGs located from 768 bp upstream of the transcription start site (TSS) to 29 bp downstream of the TSS] (Supplementary Figure S2) were generated by polymerase chain reaction (PCR) using bisulfite-treated genomic DNAs as templates. The forward and backward primers (plus tags) were: aggaagagagAGTTTTTTAAGAGGTTGAGATGGGA (forward primer for Amplicon 1), gtaatacgactcactatagggagaaggctCTTATAACTCAAAAACAACCACTCTT (reverse primer for Amplicon 1), aggaagagagGTTTTTTAGTTGGATTATGTTTTAGG (forward primer for Amplicon 2), and cagtaatacgactcactatagggagaaggctTATAATAACTCCAATTACCCTTCCC (reverse primer for Amplicon 2). The primers for Amplicon 2 were designed based on the DNA minus strand sequence of *HTR3A*. A touchdown PCR using the FastStart Taq DNA Polymerase (Roche, Mannheim, Germany) was performed for these two amplicons, including three cycles of 95°C 30 sec/66°C 15 sec/72°C 1 min, three cycles of 95°C 30 sec/64°C 15 sec/72°C 1 min, three cycles of 95°C 30 sec/62°C 15 sec/72°C 1 min, and 37 cycles of 95°C 30 sec/60°C 15 sec/72°C 1 min. After treatment with alkaline phosphatase ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), PCR products were used as a template for *in vitro* transcription followed by RNase A cleavage for the T-reverse reactions. The products were spotted on a 384-pad SpectroCHIP (Sequenom, San Diego, CA, USA) followed by spectral acquisition on a MassARRAY Analyzer. The methylation calls were performed by the EpiTYPER software v1.0 (Sequenom, San Diego, CA, USA), which generates quantitative results for each CpG site or an aggregate of multiple CpG sites. Methylation differences of *HTR3A* promoter CpGs between AD cases and controls (replication samples), the effect of sex on DNA methylation in controls, and the correlation of age and DNA methylation were analyzed as described above.

RESULTS

AD-associated DNA Methylation Changes Measured by the Illumina Methylation Array Assay

The association analysis results of 384 CpGs in 82 candidate genes are summarized in Supplementary Table S1 and CpGs with *P* values ≤ 0.001 (all AD cases *vs*. controls) are listed in Table 2. In AAs, two CpGs in two genes (*GABRB3* cg07763397 and *POMC* cg17915420) showed higher methylation levels in AD cases than in controls ($P \leq 0.001$, which did not survive Bonferroni correction). A trend toward hypermethylation of these two CpGs also appeared in AD only cases (seven AA cases with AD and no comorbid CD or OD) (*GABRB3* cg07763397: $P = 0.174$; *POMC* cg17915420: $P = 0.065$). In EAs, six CpGs in six genes (*HTR3A* cg08989585, *NCAM1* cg21572351, *DRD4* cg08079114, *MBD3* cg21372728, *HTR2B* cg27531267, and *GRIN1* cg09864658) showed higher methylation

levels in AD cases than in controls ($P \leq 0.001$); the P value of CpG cg08989585 ($P = 0.00007$) in the promoter region of *HTR3A* withstood Bonferroni correction (requiring P values to be smaller than 0.05/384 = 0.0001). Similar results were also obtained in AD only cases (68 EA cases with no comorbid CD or OD) ($P = 0.007 – 0.0005$). Moreover, methylation changes of the above eight CpGs in AD cases with comorbid CD and/or OD are presented in Supplementary Table S2.

AD-associated HTR3A Promoter Methylation Alterations Verified by Sequenom MassARRAY

Methylation levels of eight CpGs (including CpG-768 or cg08989585) in the *HTR3A* promoter region (Supplementary Figure S2) were analyzed in the replication sample (Set II) (methylation levels of CpG-738 and CpG-679 were undetectable because of the low mass of RNase A digested fragments). The six *HTR3A* promoter CpGs did not show significant methylation differences between EA cases and EA controls (all AD cases *vs*. controls: $P = 0.067 – 0.877$; AD only cases *vs*. controls: $P = 0.068 – 0.822$) (Table 2). Nevertheless, *HTR3A* CpG-768 (or cg08989585) showed non-significantly higher methylation levels in EA cases (all AD cases: 26.9%; AD only cases: 32.7%) than in EA controls (18.6%) (all AD cases *vs*. controls: $P = 0.139$; AD only cases *vs*. controls: $P = 0.093$). Methylation changes of these six *HTR3A* CpGs in AD cases with comorbid CD and/or OD are presented in Supplementary Table S2.

Influence of Sex and Age on DNA Methylation Levels

The influence of sex and age on methylation levels of CpGs that showed altered methylation in AD cases was analyzed in healthy controls. As shown in Table 3, sex significantly influenced the methylation level of *POMC* cg17915420 ($P = 0.004$) but not *GABRB3* cg07763397 ($P = 0.053$) in AAs. Moreover, EA male and EA female controls did not show significant differences in methylation levels of CpG sites in the six genes (*HTR3A*, *NCAM1*, *DRD4*, *MBD3*, *HTR2B*, and *GRIN1*) ($P > 0.05$). Additionally, the correlation of age and DNA methylation was examined but no significant effect of age on methylation levels of these CpGs was observed ($P > 0.05$) (Table 3).

DISCUSSION

In addition to epigenetic inheritance, methylation status of genes can be altered by environmental factors. The present study provided evidence that chronic alcohol consumption might influence methylation levels of a number of genes such as *HTR3A*. The exploratory study (by Illumina GoldenGate Methylation Array assays) demonstrated a hypermethylation of *HTR3A* promoter CpG cg08989585 (or CpG-768) in EA alcoholic subjects. The replication study using Sequenom MassARRAY assays showed similar results; although showing a trend toward significance, the size of the replication sample was small. The effect size of *HTR3A* promoter CpG cg08989585 (or CpG-768) in both the original and the replication samples was assessed using the Cohen's d method (Chen et al., 2012; Cohen, 1988). The effect size of *HTR3A* CpG cg08989585 (or CpG-768) in the initial set of samples was 0.454, which was a little higher than that (d=0.335) in the replication sample but in the same direction. When two sets of samples were combined and assessed using the variance inverse method, the pooled effect size was 0.423 using the Z test [Z=3.74, P(Z)=0.002]. No significant heterogeneity was observed in these two sets of samples by the Cochran' Q test [Q=0.21, P(Q) =0.646], indicating that *HTR3A* CpG cg08989585 had a similar effect size in the two sets of samples.

As described in the Introduction, several published studies have demonstrated hypermethylation of promoter CpGs of the genes, including *SNCA* (Bonsch et al., 2005),

MAOA (Philibert et al., 2008a), *SCL6A4* (Philibert et al., 2008b), *NR2B* (or *GRIN2B*) (Biermann et al., 2009), *POMC* (Muschler et al., 2010), *DNMT3B* (Bonsch et al., 2006), and *HERP* (Bleich et al., 2006) in AD cases. Five of these genes (*MAOA*, *SLC6A4*, *GRIN2B*, *POMC* and *DNMT3B*) were included in the present study. We also noted altered methylation levels of these genes in AA or EA cases ($P < 0.05$, refer to Supplementary Table S1). Although the *P* values for statistical significance obtained from CpGs of these genes did not survive Bonferroni correction, these results are consistent with previous reports.

Additionally, the effect of AD on DNA methylation appeared to be population-specific. Two CpGs in two genes (*GABRB3* and *POMC*), which were more highly methylated in AA cases than in AA controls ($P \leq 0.001$), did not show significant methylation differences between EA cases and EA controls. Conversely, six CpGs in six genes (*HTR3A*, *NCAM1*, *DRD4*, *MBD3*, *HTR2B* and *GRIN1*), which were more highly methylated in EA cases than in EA controls ($P \leq 0.001$), also did not show significant methylation differences between AA cases and AA controls (Table 2). The basis for population differences in the methylation levels of the same CpG sites as a function of AD is unknown. One possible explanation is that, similar to genetic variant allele frequency, which varies by population, methylation levels of CpG sites may be heritable. Animal studies have demonstrated that epigenetic marks established during the life of an organism can be passed on to the following generation (Probst et al., 2009). Further analyses of our data indicated that 220 (57.3%) of the 384 CpGs had significant methylation differences between AA and EA subjects ($P \leq 0.05$) (data not shown). Another possible explanation is that the patterns of drinking, smoking or drug use differ by population, leading to different DNA methylation profiles.

To verify that DNA methylation changes in cases are solely due to AD rather than comorbid CD and/or OD, DNA methylation levels of CpGs in AD only cases (i.e., AD cases with no comorbid CD or OD) and controls were compared. The above CpGs (e.g., *HTR3A* cg08989585) also showed higher methylation levels in AD only cases than in healthy controls (Table 2). We thus conclude that altered methylation of CpG sites in cases were mainly attributable to AD. Additionally, we examined whether covariates such as sex and age confounded our findings. As presented in Table 3, methylation levels of CpGs (except *POMC* cg17915420) were not significantly influenced by sex. Furthermore, age was not significantly correlated with methylation levels of CpGs. These findings provide further evidence that DNA methylation alterations in cases were associated specifically to AD.

Taken together, our data suggest that promoter DNA methylation profile is associated with AD in a population-specific way. The present study has several limitations. First, the findings derived from human peripheral blood may be biased by various types of cells in the blood (e.g., the proportion of leukocytes to non-leukocytes varies among individuals). Thus, it would be preferable to use genomic DNA extracted from sorted blood cells to conduct the experiment. Second, DNA methylation levels in blood cells may not reflect those in brain tissues. Since the rewarding effect of alcohol is mediated by the brain's reward center, it would be more appropriate to use human postmortem brain tissue (which are not easily accessible) to study DNA methylation in relation to AD risk. Mouse brain tissues could be used for a replication study. Recently, we used a mouse model study to examine ethanol-induced methylation changes in the mouse serotonin receptor 3a gene (*Htr3a*) promoter region in mouse blood and brain reward regions and similar results were obtained (Barker et al., unpublished). Third, although we analyzed a larger number of CpG sites (or genes) than published studies, given the fact that AD is a complex genetic disorder, it is likely that some genes that contribute to AD risk were missed. Additionally, the density of CpG sites detected by our customized methylation array is limited. Some functionally important CpG sites may have been missed. Therefore, a high-density methylation study of CpGs across the

genome is needed to examine the association of DNA methylation and AD. Finally, functional studies should be performed to verify whether methylation alterations in gene promoter region mediate the level of gene transcription.

In conclusion, this study is one of the first to investigate the association between DNA methylation and AD using a customized DNA methylation array-based assay. To the extent that the observed peripheral blood DNA methylation changes associated with AD reflect effects on brain tissues, our findings of differences in DNA methylation of AD-associated genes could be used as biomarkers for the prevention and treatment of AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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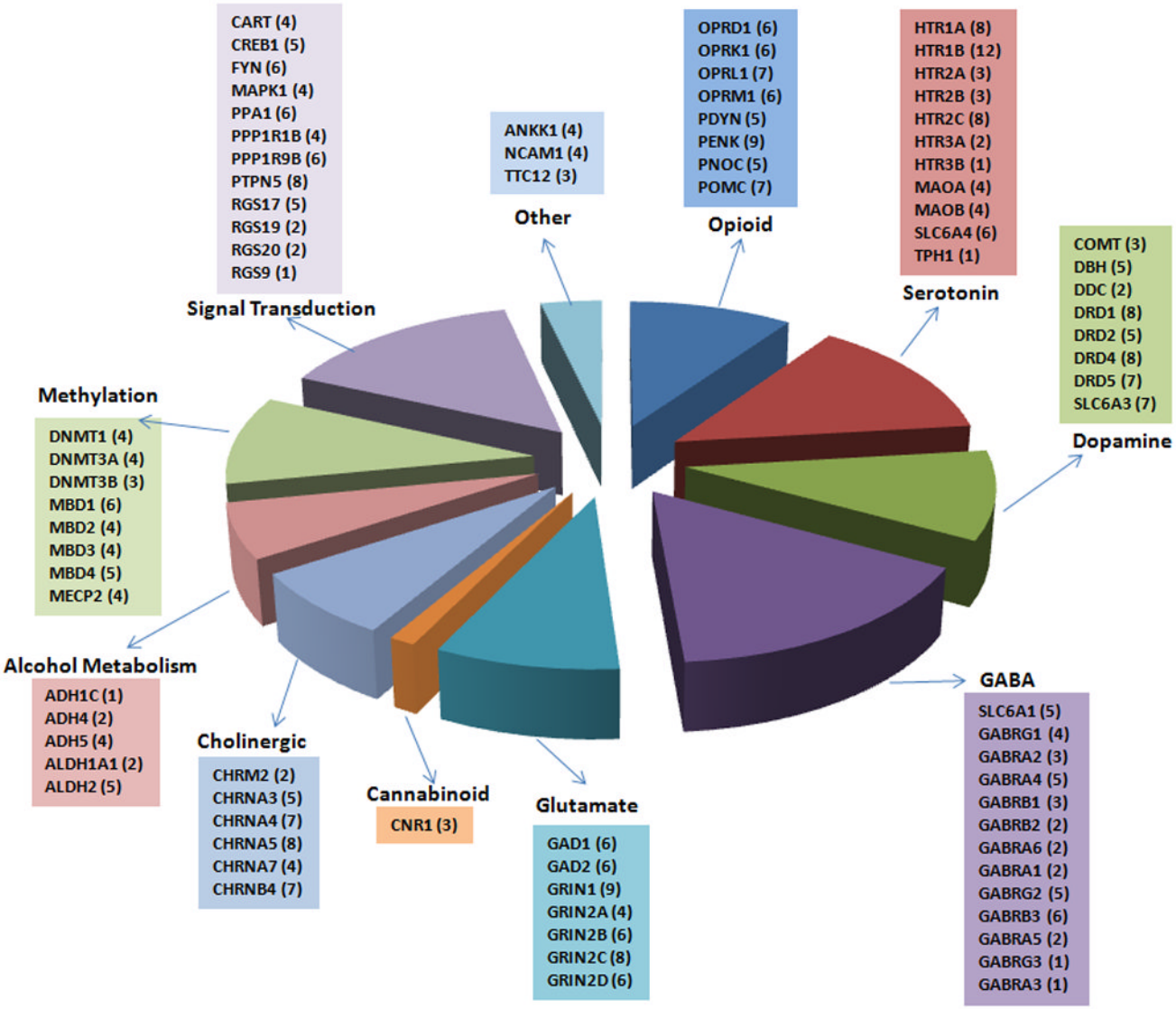

Fig. 1. 384 CpGs in 82 candidate genes

The GoldenGate methylation array consisted of 384 CpGs in 82 candidate genes. Numbers in parentheses are the number of CpG sites examined in each gene.

Table 1

Characteristics of the Study Samples

	African Americans (Set I)		European Americans (Set I)		European Americans (Set II)	
	AD Cases (n=141)	**Controls (n=144)**	**AD Cases (n=144)**	**Controls (n=105)**	**AD Cases (n=49)**	**Controls (n=32)**
AD only, n (%)	7 (5.0%)	0 (%)	68 (47.2%)	0 (%)	10 (20.4%)	0 (%)
AD+CD, n (%)	131 (92.9%)	0 (%)	71 (49.3%)	0 (%)	38 (77.6%)	0 (%)
AD+OD, n (%)	18 (12.8%)	0 (%)	29 (20.1%)	0 (%)	20 (40.8%)	0 (%)
AD+CD+OD, n (%)	16 (11.3%)	0 (%)	24 (16.7%)	0 (%)	19 (38.8%)	0 (%)
Days of intoxication in the past 30 days (mean ± S.D.)	6 ± 9	0 ± 2	5 ± 8	1 ± 2	3 ± 7	1 ± 3
	t = −7.42, $P < 0.001$		t = −5.36, $P < 0.001$		t = −1.88, $P = 0.064$	
Years of intoxication lifetime (mean ± S.D.)	11 ± 10	0 ± 1	14 ± 12	0 ± 1	12 ± 10	0 ± 0
	t = −13.85, $P < 0.001$		t = −11.51, $P < 0.001$		t = −6.64, $P < 0.001$	
Sex, Male (%)	71 (50.4%)	32 (22.2%)	75 (52.1%)	54 (51.4%)	26 (53.1%)	12 (37.5%)
	$\chi^2 = 24.43$, df = 1, $P < 0.001$		$\chi^2 = 0.010$, df = 1, $P = 0.919$		$\chi^2 = 1.88$, df = 1, $P = 0.170$	
Age, years (mean ± S.D.)	42 ± 8	37 ± 14	41 ± 12	37 ± 16	42 ± 11	37 ± 14
	t = −4.34, $P < 0.001$		t = −2.55, $P = 0.011$		t = −1.75, $P = 0.083$	

Set I sample: DNA methylation levels were quantified by Illumina GoldenGate methylation array assay.

Set II sample: *HTR3A* promoter CpG methylation levels were quantified by Sequenom EpiTYPER assay.

AD, alcohol dependence; CD, cocaine dependence; OD, opioid dependence;

AD only, AD cases with no comorbid CD or OD; AD+CD, AD cases with comorbid CD;

AD+OD, AD cases with comorbid OD; AD+CD+OD, AD cases with comrobid CD and OD.

Table 2

DNA Methylation Differences between Cases with Alcohol Dependence and Controls

African Americans (Set I)		**Controls (n=144)**	**All AD cases (n=141)**		**AD only cases (n=7)**	
CpGs	**Genes**	**Mean±SEM**	**Mean±SEM**	$P_{adjusted}$	**Mean±SEM**	$P_{adjusted}$
cg07763397	*GABRB3*	0.434±0.015	0.538±0.016	0.0005	0.536±0.034	0.174
cg17915420	*POMC*	0.276±0.006	0.311±0.006	0.001	0.322±0.015	0.065
European Americans (Set I)		Controls (n=105)	All AD cases (n=144)		AD only cases (n=68)	
CpGs	Gene	Mean±SEM	Mean±SEM	$P_{adjusted}$	Mean±SEM	$P_{adjusted}$
cg08989585	*HTR3A*	0.164±0.002	0.176±0.002	0.00007	0.177±0.002	0.002
cg21572351	*NCAM1*	0.133±0.002	0.147±0.002	0.0001	0.148±0.003	0.0005
cg08079114	*DRD4*	0.049±0.002	0.058±0.001	0.0003	0.058±0.002	0.001
cg21372728	*MBD3*	0.047±0.002	0.056±0.001	0.0004	0.056±0.002	0.007
cg27531267	*HTR2B*	0.034±0.002	0.042±0.001	0.0005	0.042±0.002	0.002
cg09864658	*GRIN1*	0.071±0.003	0.085±0.002	0.0008	0.087±0.003	0.005
European Americans (Set II)		Controls (n=32)	All AD cases (n=49)		AD only cases (n=10)	
CpGs	Gene	Mean±SEM	Mean±SEM	$P_{adjusted}$	Mean±SEM	$P_{adjusted}$
CpG-768 (cg08989585)	*HTR3A*	0.186±0.044	0.269±0.033	0.139	0.327±0.070	0.093
CpG-572	*HTR3A*	0.439±0.055	0.314±0.044	0.086	0.202±0.110	0.068
CpG-310	*HTR3A*	0.470±0.065	0.628±0.052	0.067	0.698±0.125	0.078
cpG-159	*HTR3A*	0.272±0.051	0.219±0.041	0.425	0.203±0.100	0.642
CpG-9	*HTR3A*	0.318±0.051	0.278±0.041	0.544	0.262±0.097	0.639
CpG+29	*HTR3A*	0.400±0.063	0.413±0.051	0.877	0.417±0.119	0.822

AD, all cases with alcohol dependence; AD only, AD cases with no comorbid CD or OD.

Mean±SEM, Mean methylation levels (± standard error mean) of CpGs.

$P_{adjusted}$, *P* values obtained from multivariate linear regression analysis and adjusted by covariates (sex, age, ancestry proportions, days of intoxication in the past 30 days, and years of intoxication).

GABRB3, the γ-aminobutyric acid (GABA) A receptor b3 gene; *POMC,* the proopiomelanocortin gene; *HTR3A,* the serotonin receptor 3A gene; *NCAM1,* the neural cell adhesion molecule 1 gene; *DRD4,* the dopamine receptor D4 gene; *MBD3,* the methyl-CpG binding domain protein 3 gene; *HTR2B,* the serotonin receptor 2B gene; *GRIN1,* the N-methyl-D-aspartate receptor subunit NR1 gene.

Table 3

Influence of Sex and Age on DNA Methylation in Healthy Controls

African Americans (Set I)		**Males (n=32) *vs.* Females (n=112)**			**Age**	
CpGs	**Genes**	**Males (Mean±SEM)**	**Females (Mean±SEM)**	***P***	**Pearson Correlation**	***P***
cg07763397	*GABRB3*	0.506±0.012	0.480±0.006	0.053	−0.162	0.052
cg17915420	*POMC*	0.306±0.005	0.289±0.003	0.004	−0.061	0.470
European Americans (Set I)		Males (n=54) *vs.* Females (n=51)			Age	
CpGs	Gene	Males (Mean±SEM)	Females (Mean±SEM)	*P*	Pearson Correlation	*P*
cg08989585	*HTR3A*	0.169±0.002	0.165±0.002	0.290	0.096	0.332
cg21572351	*NCAM1*	0.138±0.003	0.133±0.003	0.265	0.018	0.859
cg08079114	*DRD4*	0.052±0.002	0.049±0.002	0.143	0.023	0.812
cg21372728	*MBD3*	0.048±0.002	0.049±0.002	0.771	−0.059	0.548
cg27531267	*HTR2B*	0.037±0.002	0.033±0.002	0.147	0.061	0.533
cg09864658	*GRIN1*	0.078±0.003	0.072±0.003	0.230	−0.041	0.678
European Americans (Set II)		Males (n=12) *vs.* Females (n=22)			Age	
CpGs	Gene	Males (Mean±SEM)	Females (Mean±SEM)	*P*	Pearson Correlation	*P*
CpG-768 (cg08989585)	*HTR3A*	0.140±0.054	0.226±0.045	0.242	0.188	0.347
CpG-572	*HTR3A*	0.534±0.103	0.376±0.078	0.242	0.112	0.54
CpG-310	*HTR3A*	0.382±0.114	0.505±0.087	0.410	−0.109	0.553
cpG-159	*HTR3A*	0.197±0.089	0.286±0.068	0.445	0.246	0.175
CpG-9	*HTR3A*	0.332±0.085	0.309±0.065	0.837	−0.007	0.971
CpG+29	*HTR3A*	0.351±0.110	0.430±0.084	0.581	−0.123	0.503

β±SEM, Mean methylation levels (± standard error mean) of CpGs.

P, P values obained from t-tests (or Pearson correlation tests).

GABRB3, the g-aminobutyric acid (GABA) A receptor b3 gene; *POMC,* the proopiomelanocortin gene; *HTR3A,* the serotonin receptor 3A gene; *NCAM1,* the neural cell adhesion molecule 1 gene; *DRD4,* the dopamine receptor D4 gene; *MBD3,* the methyl-CpG binding domain protein 3 gene; *HTR2B,* the serotonin receptor 2B gene; *GRIN1,* the N-methyl-D-aspartate receptor subunit NR1 gene.