Epigenetic Profiling of DNA Methylation Changes Associated With Chronic Alcohol Consumption: a 12-year Follow-up Study

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Abstract—**Alcoholism has always been a major public health concern in Taiwan, especially in the aboriginal communities. DNA methylation has recently been found to be associated with alcoholism. Since 1986, we have been following up on the mental health conditions of four major aboriginal peoples of Taiwan. In the current study, we attempted to profile the effect of chronic alcohol exposure on the epigenome. Clinical interviews were performed on 993 aboriginal people at phase 1 (1986), and followed up through phase 2 (1990-1992), and phase 3 (2003-2009) with DNA preparations at phases 2 and 3. Selected individuals for the present study included males from the phase 1 normal cohort who remained normal at phase 2 and became dependent on alcohol by phase 3 (n=10) and control subjects that have not had any drinking problems throughout the study (n=10). We assessed changes in DNA methylation in the blood collected at phases 2 and 3. Preliminary data show that 201 and 254 genes contain sites that are differentially methylated between the two collection time points in the control and case subjects, respectively. Among the list of genes differentially methylated in the case group, the methylation levels of 6 genes were found to correlate with alcohol consumption. These include genes involved in neurogenesis (NPDC1) and inflammation (HERC5) as well as alcoholism-associated genes ADCY9, CKM, and PHOX2A. Our study identified genes that are associated with chronic alcohol consumption at the epigenetic level. The results offer a comprehensive epigenomic map that helps enhance our understanding of alcohol-induced damages.**

Keywords- alcoholism; epigenetic profiling; DNA methylation; longitudinal study; Taiwanese aboriginals

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I. INTRODUCTION

Multiple genetic factors determine an individual's predisposition to alcohol use disorder (AUD) [1-3]. Approximately 40 to 60% of the variance in the development of alcohol dependence is attributable to the genotype [4, 5]. As dependence develops only following repeated use, the neurobiological changes that arise in response to repeated alcohol exposure are the key factors interplaying between the genotype and the environment [6].

Alcohol exposure is known to cause aberrant DNA methylation [7-9], which is the best understood epigenetic modification of gene expression. The severity of epigenetic changes can be aggravated by alcohol consumption [9]. Understanding the epigenetic process of chronic alcohol use may represent a potential therapeutic target for compulsive drinking.

Emerging evidence supports the involvement of DNA methylation changes in alcoholism. For example, alpha synuclein, a gene associated with dopaminergic neurotransmission, showed promoter DNA hypermethylation in the peripheral mononuclear cells of alcohol dependent patients [10]. Altered DNA methylation has been found in the promoters of the atrial natriuretic peptide, vasopressin, and N-methyl-D-aspartate 2b receptor subtype genes in patients undergoing alcohol withdrawal [11, 12]. Hypermethylation of the dopamine transporter gene has been associated with alcohol

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dependence [13]. Methylation of the CpG sites in the promoter region of the polypeptide pro-opiomelanocortin is different between alcohol-dependent and healthy controls and linked to craving [14]. These studies indicate that changes in DNA methylation may contribute to the complex spectrum of AUD. A genome-wide approach has the potential to uncover the various epigenetic signatures of alcoholism.

In this study, we assessed DNA methylation changes of long-term alcohol consumption among subjects from a longitudinal study of AUD in the Taiwan Aboriginal Study Project (TASP) [15]. TASP began in 1986 (N=993) with four major Taiwanese aboriginal groups (Ami, Atayal, Paiwan, and Bunun). In the phase 1 cross-sectional survey (1986-88), high lifetime prevalence rates of DSM-III-R AUD were found among the four groups, ranging from 44.5% to 54.5% [16]. In the phase 2 follow-up conducted four years later (1990-92), the age-standardized annual incidence rates of AUD among the four groups were found to range from 2.8 to 4.9% [17]. A phase 3 16-year follow-up was conducted in 2003-2009. The blood samples collected at phases 2 and 3 with 12 years in between presented a great opportunity to examine the effect of alcohol drinking on DNA methylation among incidence cases of AUD during the period. Here, we report a genome-wide profiling of DNA methylation changes associated with chronic alcohol consumption, providing an epigenomic picture that help enhance our understanding of alcohol-induced damages.

II. METHODS AND MATERIALS

A. Participants

All procedures were reviewed and approved by the Institutional Review Board of Academia Sinica, Taiwan, and written informed consents were obtained from all participants. For those who had reduced ability to consent, the carers or guardians gave written informed consent on behalf of these participants. Our analysis was based on a subset of participants. Because existing evidence suggests that DNA methylation may be gender specific [18, 19], this study only focused on male cohort subjects as a preliminary investigation into the association between DNA methylation and alcohol drinking.

Several criteria were considered for the selection of cases. Since DNA preparation was performed at phases 2 and 3, study subjects must have DNA samples available from both phases. The amount and the quality of these samples must meet the requirements for array analysis as well. Also, to study the effect of alcohol on methylation patterns, we chose AUD cases with no lifetime drinking problem at phase 2, but had developed AUD after phase 2 and were diagnosed with DSM-IV alcohol dependence at phase 3. Moreover, these subjects must have been heavy drinkers ($\geqslant$ 80g of alcohol intake/day) at phase 3 when their blood samples were collected. All subjects from the male cohort fulfilling these criteria have been included (N=10). Every case was matched in age ($\pm$3 years) with one male control who had never been found to have lifetime DSM-IV alcohol dependence throughout the three waves of investigations.

B. Methylation analysis

For each individual, 10 ml of blood was drawn at phases 2 and 3. Blood samples were collected into BD Vacutainer® SST™ blood collection tubes (Franklin Lakes, NJ, USA). DNA extraction was performed using the Gentra Puregene Blood Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. DNA quality and quantity were determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Techonologies, Wilmington, DE, USA). DNA integrity was evaluated by agarose gel electrophoresis. 500 ng of each sample underwent bisulfite conversion using the EZ DNA methylation kit from Zymo Research (Orange, CA, USA) following the manufacturer's protocol with modifications recommended by Illumina (San Diego, CA, USA). To confirm that bisulfite conversion was successful, quality control PCR reactions were performed for each sample using the primers and protocol described by Thirlwell et al. (2010) [20]. Bisulfite converted DNA samples were then subjected to methylation profiling on the Infinium® HumanMethylation27 BeadChips (Illumina Inc., San Diego, CA, USA) according to Illumina's instructions at Genetech Biotech in Taipei, Taiwan. Each locus was interrogated by a specific probe linked to a methylated (M) and unmethylated (U) bead type. Fluorescent intensities of the M and U beads were used to calculate the methylation status of a specific CpG site, following the formula: ß=M/((M+U)+100).

C. Statistical analysis

Comparisons of variables such as age, alcohol consumption, smoking and betel nut use were performed using the Mann-Whitney test in SPSS v18.0 (Somers, NY, USA). For methylation comparison, data were background normalized in Illumina's BeadStudio (San Diego, CA, USA) and exported for additional analysis using GeneSpring® (Agilent Technologies, Santa Clara, CA, USA). Our analysis was based on genes corresponding to CpG sites (or probes) that were methylated or unmethylated. Average beta values for the CpG sites in the case and control groups were calculated. In our data, there were genes with two or more sites showing similar results. These duplicate records were removed so that the same genes were not counted twice.

Differential methylation between phases 2 and 3 in the case and control was analyzed using Wilcoxon paired sample test. Differential methylation at a particular CpG site was considered to be significant if $p<0.01$. Neither the case nor the control group was genotyped for the CpG sites. Since it was possible that genotype may affect methylation, we limited our analysis to identifying methylation differences that occurred within each individual from phase 2 to phase 3, and not between the case and control groups. However, significantly differentially methylated genes (or CpG sites) in the control group were used to identify potential aging-related changes in the case group. The remaining differentially methylated genes were then analyzed for correlation with alcohol consumption using Spearman's correlation test.

D. Bioinformatics analysis

We used MetaCore (GeneGo, St. Joseph, MI) to analyze significantly differentially methylated genes. Gene set enrichment analysis was performed to evaluate the most

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important biological processes, molecular functions, and networks. For a network of a given size, MetaCore can be used to calculate statistical significance based on the probability of the network's assembly from a random set of nodes (genes) and the same size as the input list (P value).

III. RESULTS

The case and control groups appeared to be similar in the distribution of age, education, mental disorders, and average alcohol consumption at phases 1 and 2 (Table 1). Average monthly alcohol consumption at phase 3 was significantly much higher in the case than in the control group. The use of betel nut and cigarette was only enquired at phase 3. Only cigarette smoking showed a significant difference between the case and control group at phase 3 (Table 1: Mann Whitney U=26.0, p=0.055 for betel nut; Mann Whitney U=26.5, p=0.045 for cigarette smoking). As we only have detailed information regarding alcohol consumption in all phases of the study and the average amount of cigarette consumed in both groups appeared to be small, we speculated that alcohol contributed strongly to the differences between the case and control group. Hence, our analysis was focused on the relationship between alcohol and DNA methylation.

We determined the number of shared and group-specific differentially methylated genes in the normal and alcohol-dependent individuals for each phase (Fig. 1). Among the 27,578 CpG sites interrogated in this study, 252 (68 up- and 184 down-regulated genes) and 200 (149 up- and 51 down-regulated genes) were found to be in the control and case group, respectively. The two groups shared three differentially methylated genes. Interestingly, there appeared to be genome-wide down-regulation of methylation in the control group. Yet, in the case group, there seemed to be global hypermethylation.

The differentially methylated genes identified from our study have diverse molecular roles, including signal transduction, intracellular trafficking, stress and immune responses, structural maintenance, as well as regulation of metabolism and transcription.

We performed bioinformatics analysis using Metacore to identify the relevant biological pathways that are important in the case and control groups (Fig. 2). The genes that were differentially methylated in the control group seemed to be more enriched in the categories under visual perception,

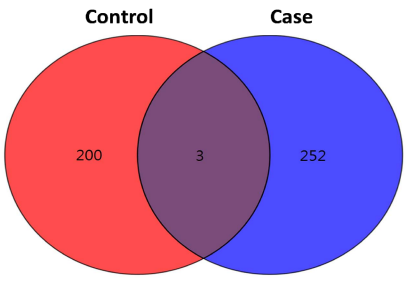

Figure 1. Number of genes differentially methylated in the control and case group. Red and blue represent control- and case-specific differentially methylated genes, respectively. Purple indicates genes that are differentially methylated in both cases and controls.

TABLE I. SOCIO-DEMOGRAPHIC CHARACTERISTICS, USE OF CIGARETTE AND BETEL NUT, AND PSYCHIATRIC DIAGNOSES IN THE CASE AND CONTROL GROUPS OF ALCOHOL USE DISORDER AT THREE PHASES DURING THE FOLLOW-UP.

	Controls N=10	Cases N=10	Non-parametric statistics (*p*-value)
Age	40 ± 2	39 ± 2	0.36
Education (years)	11 ± 2	10 ± 3	0.37
Phase 1 average alcohol consumption (units/month)	4.3 ± 1.9	15.8 ± 6.8	0.30
Phase 2 average alcohol consumption (units/month)	19.3 ± 12.0	31.1 ± 9.47	0.40
Phase 3 average alcohol consumption (units/month)	39.5 ± 1.44	589 ± 122	0.00
Phase 3 betel nut use (number/month)	2 ± 1	5 ± 1	0.055
Phase 3 cigarette smoking (number/month)	3 ± 1	6 ± 1	0.045
Depressive disorders, N	2	4	0.08
Anxiety disorders, N	2	3	1.00
Adjustment disorder, N	1	0	0.748

One unit of alcohol is defined as 12 grams of alcohol. Anxiety disorders include generalized anxiety disorder, panic disorder, posttraumatic disorder, and anxiety disorder, NOS. Depressive disorders include major depressive disorder and dysthymic disorder.

TABLE II. CORRELATION OF DIFFERENTIALLY METHYLATED GENES WITH AVERAGE MONTHLY ALCOHOL CONSUMPTION IN CASE SUBJECTS OVER THE 12 YEARS BETWEEN PHASES 2 AND 3.

	Gene ID	Product	Correlation
ADCY9	115	adenylate cyclase type 9	0.60
TMEM45B	120224	transmembrane protein 45B	0.59
PHOX2A	401	paired-like homeobox 2a	0.70
HERC5	51191	HECT domain and RLD 5	0.66
CKM	1158	muscle creatine kinase	-0.56
NPDC1	56654	neural proliferation, differentiation, and control, 1	-0.67

Pearson correlation test, $p<0.01$

immune response, cell adhesion and differentiation. In contrast, those in the alcoholic group appeared to be involved in functional pathways responsible for molecular and cellular structural maintenance, lipid metabolism, glucocorticoid receptor signaling, apoptosis and oxidative stress, as well as atherosclerosis associated gene regulation.

We constructed a gene-gene interaction network and correlation analysis of the differentially methylated genes to identify changes in methylation that may be associated with alcohol consumption. The interaction network identified the possible dynamics underlying alcohol-induced damages (Fig. 3). Correlation analysis also showed that several genes involved in cellular stress pathways were associated with average monthly alcohol consumption over the 12 years between phase 2 and phase 3 (Table 2).

IV. DISCUSSION

Based on a longitudinal follow-up study of AUD in Taiwanese aborigines, our findings reveal genome-wide hypermethylation associated with alcohol dependence, consistent with other studies [10, 12, 13, 21]. Further, we have identified several specific epigenetic signatures associated with chronic alcohol consumption. Compared to the control group, most of the differentially methylated genes in the case group, are involved in lipid metabolism and atherosclerosis regulation,

cellular and molecular structural integrity, and cellular stress pathways. Our results are in line with the observations that chronic alcohol exposure leads to alterations in membrane structures [22], as well as actin and microtubule organization [23]. Several studies have linked heavy alcohol consumption with a higher risk of atherosclerosis and/or fatty liver [24]. The present finding implicates the possible epigenetic mechanisms underlying chronic alcohol drinking and the development of physical disorders.

Our gene network analysis indicates that the differentially methylated genes in the alcoholic individuals are involved in complex interactions with each other (Fig. 3). Most of these associations are linked through two alcoholism-related genes: androgen receptor (AR) and arginine vasopressin protein (AVP). AR is known to have a modulating effect on craving during withdrawal in men [25], whereas AVP is associated with alcohol tolerance [26]. Connected to the AR and AVP are genes involved in aldehyde detoxification (ALDH1A3) [27], regulation of circadian clock (PROK2) [28], maintenance of cell adhesion (MADCAM1) [29], as well as the development of various types of cancer, including MYBL2 [30], CRYAB [31], CDKN2B [32], etc. The potential interactions among these differentially methylated genes suggest that the effect of alcohol on DNA methylation is complex. Alcohol-induced changes in methylation may modulate the susceptibility to cancer, affect aldehyde metabolism, alter cellular physiology, and moreover, regulated behavior.

Some of the differentially methylated genes in the case group are related to the various characteristics of AUD. For instance, AR is a craving mediator [25]. Creatine kinase (brain: CKB or muscle: CKM) has been associated with alcohol dependence, withdrawal and delirium tremens [33]. Our results show that methylation of the CKM gene also appears to be negatively correlated with alcohol consumption. The tachykinin receptor 1 genotypes have been implicated in craving and severity of alcohol dependence. Moreover, the level of AVP is known to fluctuate with alcohol consumption, modulating craving, tolerance and alcohol-induced memory impairment [12, 34]. Our observation that these genes are differentially methylated in the alcoholic group and potentially interacting with each other indicates that DNA methylation may represent a possible mechanism underlying AUD.

Most of the differentially methylated genes in the case group are associated with lipid metabolism and atherosclerosis regulation, cellular and molecular structural integrity, and cell stress pathways. These biological processes are particularly sensitive to alcohol-induced damages. Our results are in line with the observations that chronic alcohol exposure leads to genome-wide hypermethylation, leading to alterations in membrane structures [22], actin and microtubule organization [23]. Several studies have also linked heavy alcohol consumption with a higher risk of atherosclerosis and/or fatty liver [24].

Correlation analysis showed that several genes appear to be associated with the level of alcohol consumption. For example, adenylate cyclase type 9 (ADCY9) belongs to a family of transmembrane proteins involved in learning and memory, synaptic plasticity, and neurodegeneration, in addition to having been implicated in the development of addiction and mood disorders [35, 36]. Several members of the adenylate cyclase family are ethanol responsive [37]. This is consistent with our observation that chronic alcohol consumption appears to be associated with increased methylation of the ADCY9 gene. In addition, paired mesoderm homeobox protein 2A, PHOX2A, is a transcription regulator of the alpha 3 nicotinic acetylcholine receptor subunit gene [38], whose overexpression has been shown to reduce alcohol intake in mice [39]. It seems that the nicotinic acetylcholine receptor may modulate alcohol drinking behavior through PHOX2A. That methylation of PHOX2A appears to be positively correlated with the amount of alcohol consumption offers an additional evidence for the association between PHOX2A and alcohol dependence.

Some of the genes correlated with chronic alcohol consumption have been implicated in cell damage and inflammatory response. For example, methylation of the HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5) is a regulator of inflammatory response [40]. Increased methylation of HERC5 after chronic alcohol

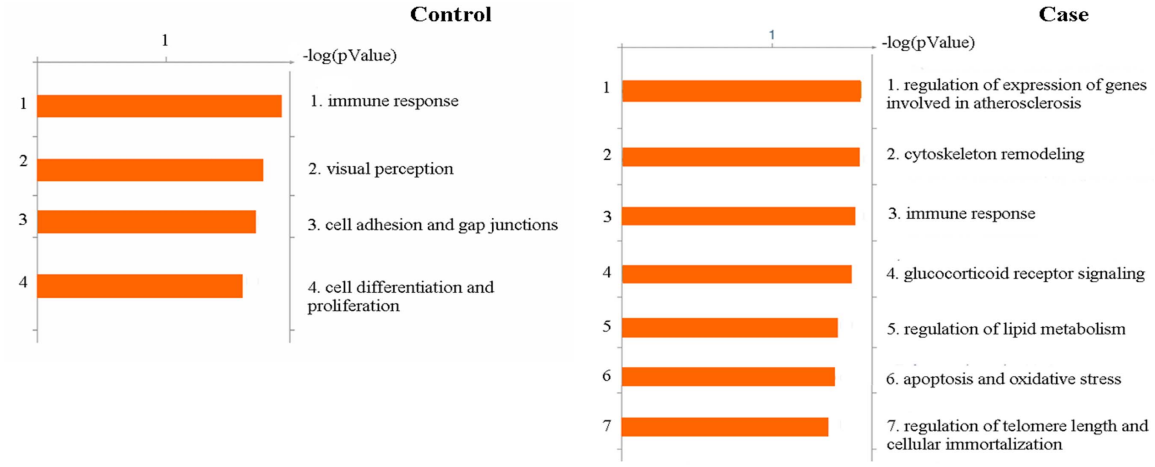

Figure 2. Gene set enrichment analysis of differentially methylated genes in the control and case group.

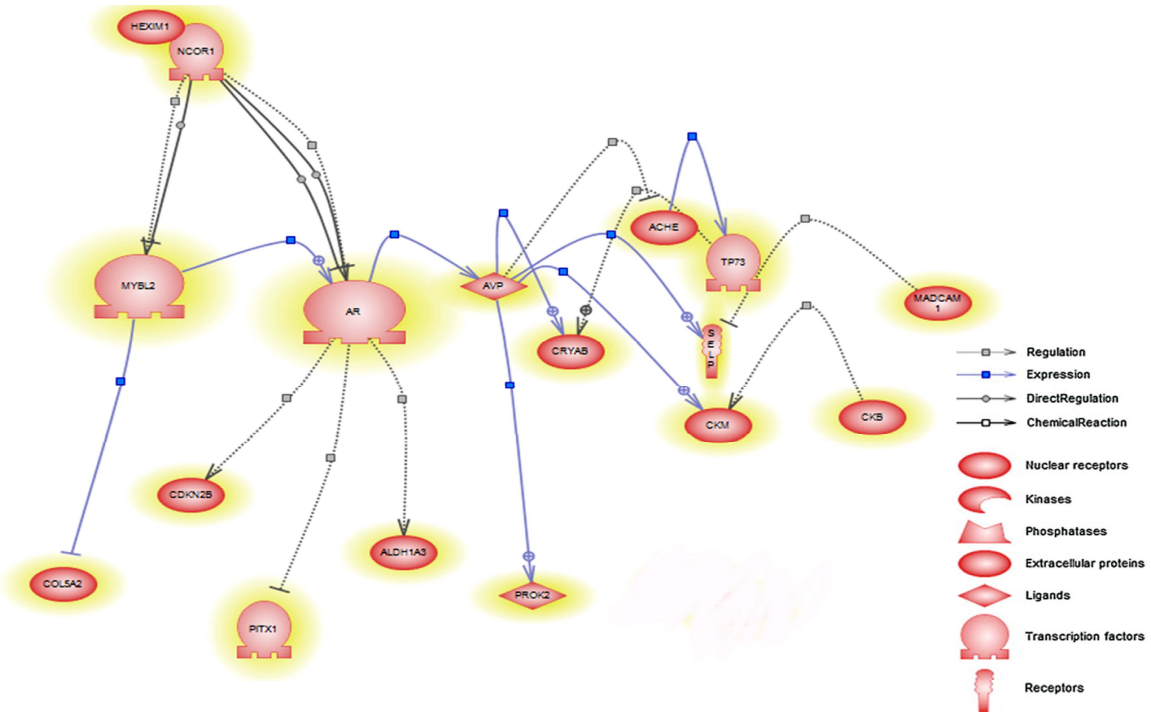

Figure 3. Potential interactions among the significantly differentially methylated genes in the case group using Ariadne Pathway Studio 7.1.

exposure suggests that alcohol may attenuate HERC5's ability to maintain a proper inflammatory response. NPDC1 (neural proliferation, differentiation, and control, 1) is a neural factor controlling neural cell proliferation and differentiation [41]. Alcohol use disorder can result in alcohol-related brain damage, but the exact mechanism of this process is not well understood. Given NPDC1's role in neurogenesis and its de-methylation with respect to chronic alcohol consumption, it is possible that there exists a protective mechanism to compensate alcohol's inhibiting effect on cell proliferation.

Our results bear relevant biological significance in the context of chronic alcohol exposure and alcohol-induced damages, but several limitations underlie our study. First, our findings, though based on a small sample size of Taiwanese aboriginal males, are yet preliminary. It is likely that we might have identified male-specific epigenetic markers unique to Taiwanese aborigines or overlooked genes of moderate effect. Second, our analysis may not fully reflect the changes in gene expression and further confirmation at the RNA level is required. Third, our epigenetic profiling in the blood may not directly represent the situations in other tissues. Finally, the lifetime frequencies and amount of cigarette smoking and betel nut use were assessed at phase 3 only. Although the average amount of cigarettes and betel nuts consumed by the study subjects appeared to be low in both cases and controls, we could not entirely eliminate the possibility that cigarette and betel nut may also modulate DNA methylation.

Collectively, our study identified genes associated with chronic alcohol consumption at the epigenetic level. Though various questions remain unanswered, by presenting a comprehensive epigenomic map of a longitudinal follow-up study on alcohol use disorder, we have provided support for the emerging notion that epigenetics plays a role in alcoholism etiology and facilitate the effort of research in this area.

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