

# Differential Expression of miR-130a in Postmortem Prefrontal Cortex of Subjects with Alcohol Use Disorders

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## Abstract

**Background:** Emerging evidence suggests that neuroadaptations to alcohol may result from chronic alcohol consumption-induced expression changes of microRNAs (miRNAs) and their target genes. Studies with animal or cell culture models have demonstrated that ethanol exposure leads to miRNA expression alterations. However, there is limited information on miRNA expression in the brains of subjects with alcohol use disorders (AUDs). The present study aimed to analyze expression changes of miRNAs and their target genes in postmortem prefrontal cortex (PFC) of AUD subjects.

**Methods:** Genome-wide miRNA and mRNA expression was examined in postmortem PFC of 23 European Australia AUD cases and 23 matched controls using the Illumina HumanHT-12 v4 Expression Bead Chip array, which targets 43,270 coding transcripts and 3,961 non-coding transcripts (including 574 miRNA transcripts). Multiple linear regression analysis and permutation test were performed to identify differentially expressed miRNAs and their target mRNAs. Target gene prediction, Gene Set Enrichment Analysis (GESA), and DAVID functional annotation clustering analysis were applied to identify AUD-associated gene sets and biological modules.

**Results:** Two miRNAs and 787 coding genes were differentially expressed in the PFC of AUD cases [miR-130a (downregulated):  $P_{\text{permutation}}=0.023$ , miR-604 (upregulated):  $P_{\text{permutation}}=0.019$ , coding genes:  $1.6 \times 10^{-5} \leq P_{\text{permutation}} \leq 0.05$ ; but all  $P$  values did not survive multiple-testing correction]. GESA showed that the 202 predicted target genes of miR-130a were highly enriched in differentially expressed genes ( $P_{\text{nominal}} < 0.001$ ), but not the 116 predicted target genes of miR-604 ( $P_{\text{nominal}} = 0.404$ ). DAVID functional clustering further revealed that the hub target genes (e.g., *ITPR2* and *ATP1A2*) of miRNA130a were mainly responsible for regulating ion channel function.

**Conclusion:** This study provides evidence that downregulation of miR-130a may lead to altered expression of a number of genes in the PFC of AUD subjects. Further studies are warranted to confirm these findings in replication samples and other reward-related brain regions.

**Keywords:** MicroRNA; Alcohol use disorders; Postmortem prefrontal cortex; Expression microarray

## Introduction

Alcohol use disorders (AUDs), including abuse or dependence, are prevalent health and social problems. Despite the high prevalence (over 8%) of AUDs [1], their molecular mechanism is not well understood; it is known that AUDs are influenced by multiple genes and gene-environment interactions [2]. Environmental factors alone (e.g., chronic alcohol consumption) may also lead to alcohol tolerance or dependence through neuroadaptations. Emerging evidence suggests that alcohol-induced neuroadaptations are at least partially linked to altered expression of microRNAs (miRNAs) and their target genes that are involved in AUD-relevant biological pathways [3].

miRNAs are a group of small non-coding RNAs (~19 - 24 nucleotides) that regulate the expression of protein-coding genes. The regulatory function of miRNAs is accomplished through the RNA-induced silencing complex (RISC). miRNAs guide the RISC to the 3' untranslated regions (3' UTRs) of their target mRNAs via base pairing, leading to translation inhibition or mRNA degradation [4,5]. miRNAs are abundant in the brain and participate in many biological processes such as neuronal differentiation [6], synapse formation and plasticity [7,8], and neurodegeneration [9]. Chronic alcohol consumption may alter miRNA expression in the brain, and dysregulation of miRNAs may lead to diseases such as AUDs. Studies with cell culture models demonstrated that alcohol-induced miRNA alterations were associated

with alcohol tolerance (in rat striatal primary neurons) [10], gut leakiness (in the human intestinal epithelial cell line Caco-2) [11], and neural proliferation and differentiation (in fetal mouse cerebral cortex-derived neurosphere) [12]. Moreover, Guo et al. [13] reported that chronic intermittent ethanol exposure or removal induced different miRNA expression patterns in mouse cortical primary neuronal cultures. Studies with a mouse model also showed that ethanol exposure caused dysregulation of specific miRNAs, which target a number of genes that are crucial in the context of fetal alcohol spectrum disorders (FASDs) [14].

To date, there is limited information on miRNA expression alterations in the brains of human AUD subjects; to our knowledge, only one study examined AUD-associated miRNAs in postmortem

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prefrontal cortex (PFC) of human AUD subjects [15]. This study identified 35 miRNAs that were upregulated in the PFC of AUD subjects, and those genes potentially targeted by the upregulated miRNAs were overrepresented by biological processes such as apoptosis, cell cycles, cell adhesion, and nervous system development. Given the relatively small size of the PFC tissue sample (14 cases vs. 13 controls) in that study, additional studies with larger samples are warranted. In the present study, we examined genome-wide gene expression in postmortem PFC of 23 AUD cases and 23 matched controls using the microarray approach, and the differentially expressed miRNAs and mRNAs in AUD cases were identified. In addition, the enrichment of the putative target genes of AUD-associated miRNAs in differentially expressed genes was also analyzed.

## Materials and Methods

### Study subjects

Autopsy brain tissue samples were obtained from the New South Wales Tissue Resource Centre (NSW TRC) at the University of Sydney. The NSW TRC is sponsored by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) for collecting human brain tissues for alcohol-related research. It has ethics approval from the Sydney Local Health Network and The University of Sydney. Fresh-frozen sections of Brodmann area 9 (BA9, mainly the dorsolateral PFC of the brain) were obtained from 23 European Australian AUD cases and 23 matched controls. Case and control samples were matched by race, sex, age, brain pH, brain weight, and postmortem interval (PMI). Cases were affected with alcohol abuse or dependence (with no comorbid drug abuse or dependence or major psychotic disorders such as schizophrenia and bipolar disorder) according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders 4<sup>th</sup> Edition (DSM-IV) [16]. Controls were assessed to be free of substance (alcohol or drug) abuse or dependence or major psychotic disorders. The case group included 23 AUD subjects and 16 (69.6%) were males. The average age of AUD cases was  $56 \pm 9$  years (mean  $\pm$  S.D.) and the average amount of daily use of alcohol in AUD cases was  $165 \pm 81$  gram. The control group included 23 matched healthy subjects and 16 (69.6%) were males. The average age of controls was  $56 \pm 9$  years and the average amount of daily use of alcohol in control subjects was  $11 \pm 9$  gram.

### Transcriptome analysis by microarray-based assay

Total RNA was extracted from postmortem PFC tissue using the miRNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Total RNA was quantified by NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA integrity number (RIN) was determined using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RIN ranged from 5 to 7 for the 46 RNA samples. Genome-wide gene expression levels were quantified using the Illumina HumanHT-12 v4 Expression BeadChip (Illumina Inc, San Diego, CA, USA). To avoid potential batch effects in expression array-based assays, biotin-labeled cDNAs generated from total RNA samples of six AUD cases and six matched controls were loaded onto the same BeadChip (i.e., 12 samples per Chips; in total, four chips were used for the 46 RNA samples). Each array targets 43,270 coding transcripts and 3,961 non-coding transcripts (including 574 miRNA transcripts). Probe intensity and gene expression data were analyzed using Illumina GenomeStudio software V2011.1 (Gene Expression Module V1.9.0).

Low-level analysis of microarray data was performed in R 2.15.1 (<http://www.r-project.org/>) using the Bioconductor package *lumi*.

The variance stabilizing transformation (VST) method and the robust spline normalization (RSN) method were applied to all arrays. After normalization, annotated genes with intensities indistinguishable from background noise (detection  $p$  value  $> 0.05$ ) in more than half of the RNA samples were removed. The R package *sva* was applied to remove batch effects directly.

### Differential expression analysis of miRNAs and mRNAs

Genome-wide gene expression alterations in postmortem PFC of AUD cases were examined using multiple linear regression analysis with adjustment for covariates such as sex, age, and PMI. To exclude false positive signals by chance, a permutation test with a million iterations was employed by use of the R package *lmPerm* for multiple linear regression analyses, in which the AUD status of each subject was relabeled randomly in each test. The  $q$ -value was computed for each nominal  $P$  value by controlling the false discovery rate (FDR) at 0.05 using the R package *qvalue* [17]. All statistical analyses were implemented using the statistical package R (version 2.15.1) (<http://www.r-project.org/>).

### miRNA-mRNA expression correlation analysis and miRNA target prediction

Given that miRNAs potentially suppress the expression of their target genes, Pearson correlation analysis was applied to identify mRNAs that were negatively correlated with differentially expressed miRNAs in their expression levels using gene expression data from the control subjects. The obtained miRNA-mRNA pairs were subsequently validated by two integrative bioinformatics programs: miRWalk [18] and miRecords [19]. For each differentially expressed miRNA, we obtained a list of putative target genes that were predicted by at least two independent programs under default settings.

### Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed as described previously [20,21]. 14,950 genes with expression levels detectable by the microarray approach were ranked by the direction of expression changes (up- or downregulated) and  $-\log_{10}(p \text{ values})$  that reflected gene expression differences between AUD cases and matched controls. Then the list of pre-ranked genes and the list of predicted target genes of differentially expressed miRNAs were used to run GSEA. A set of miRNA target genes that were enriched in differentially expressed genes was obtained.

## Results

### Differentially expressed miRNAs and mRNAs in AUD cases

Two miRNAs showed differential expression in AUD cases [miR-130a (downregulated): regression coefficients = -0.05,  $P_{\text{permutation}} = 0.023$ ; miR-604 (upregulated): regression coefficients = 0.05,  $P_{\text{permutation}} = 0.019$ ], but neither  $P$  values survived multiple-testing correction ( $q > 0.05$ ). Additionally, we found that 787 coding genes were differentially expressed (351 were upregulated and 436 were downregulated) in AUD cases ( $1.6 \times 10^{-5} \leq P_{\text{permutation}} \leq 0.05$ ), but none of these  $P$  values survived multiple-testing correction ( $q > 0.05$ ).

### Expression correlation of miR-130a (or miR-604) and its target mRNAs

Among all the genes ( $n = 14,950$ ) that were detectable by the microarray approach, 467 genes showed negative correlations with miR-130a in their expression levels ( $P_{\text{correlation}} < 0.05$ ), and 327 genes showed negative correlations with miR-604 in their expression levels

( $P_{\text{correlation}} < 0.05$ ). With the use of two prediction programs miRWalk and miRecords, out of the 467 genes showing negative correlation with miR-130a in gene expression, 202 genes were predicted to be putative targets of miR-130a; out of the 327 genes showing negative correlation

with miR-130a in gene expression, 116 genes were predicted to be putative targets of miR-604.

### Gene set enrichment analysis of miR-130a and miR-604 target genes

We further analyzed the association of the 202 predicted target genes of miR-130a (or 116 predicted target genes of miR-604) with AUDs using gene set enrichment analysis (GSEA). The pre-ranked [by  $-\log_{10}(p\text{-value})$ ] list of all detectable genes (i.e., 14,950 genes) and the list of the 202 predicted target genes of miR-130a (or the list of the 116 predicted target genes of miR-604) were uploaded into the program GSEA to analyze whether the predicted target genes of miR-130a or miR-604 were overrepresented by the differentially expressed genes. As shown in Figure 1, the predicted target genes of miR-130a were overrepresented by those genes that were differentially expressed in AUD cases ( $P_{\text{nominal}} < 0.001$ ), and 77 of the 202 predicted target genes of miR-130a were found to be the leading genes for obtaining the maximum enrichment score. However, the predicted target genes of miR-604 were not enriched by the differentially expressed genes ( $P_{\text{nominal}} > 0.05$ ).

### Functional annotation of putative target genes of miR-130a

Among the 202 putative target genes of miR-130a, 24 (11.9 %) showed differential expression in AUD cases, and 23 of the 24 genes were upregulated in AUD cases at  $P_{\text{permutation}} \leq 0.05$  (Table 1). The proportion (23/202) of upregulated putative target genes of miR-130a was significantly higher than that of the set of all upregulated genes among all genes detected (351/14,950) (hypergeometric test:  $p = 4.0 \times 10^{-10}$ ). We further examined the correlation between the expression levels of these 23 upregulated genes and the downregulated miR-130a in all 46 samples (23 cases + 23 matched controls) with adjustment for covariates (AUD status, sex, age, and PMI). Five (*ITPR2*, *JARID2*, *C10orf54*, *BAG3*, and *TUBB2B*) of the 23 upregulated genes showed significant negative correlations with miR-130a (downregulated) in their expression levels. Moreover, *ITPR2* and *JARID2* were among the top genes ( $P_{\text{permutation}} < 0.010$ ) showing differential expression in postmortem PFC of AUD cases (*ITPR2*:  $P_{\text{permutation}} = 1.6 \times 10^{-3}$ ; *JARID2*:  $P_{\text{permutation}} = 9.8 \times 10^{-3}$ ) (Figure 2). To explore the biological function of the above 23 upregulated genes, we uploaded the list of these 23 genes

Genes	Expression (controls) <sup>a</sup>	Expression (AUD cases) <sup>a</sup>	Effect size <sup>b</sup>	$P_{\text{nominal}}$ <sup>c</sup>	$P_{\text{permutation}}$ <sup>d</sup>
<i>FBXO21</i>	9.47	9.59	0.12	0.001	0.002
<i>ITPR2</i> <sup>e</sup>	6.72	6.82	0.12	0.002	0.002
<i>FAM65B</i>	7.21	7.37	0.15	0.005	0.004
<i>ZFYVE21</i>	10.06	10.26	0.23	0.006	0.006
<i>BBS2</i>	9.17	9.31	0.18	0.005	0.007
<i>GPR177</i>	7.78	7.99	0.23	0.007	0.008
<i>JARID2</i> <sup>e</sup>	8.27	8.41	0.17	0.011	0.010
<i>RAB34</i>	7.05	7.34	0.29	0.012	0.011
<i>C10orf54</i> <sup>e</sup>	6.89	7.25	0.31	0.012	0.012
<i>SPON1</i>	8.02	8.43	0.43	0.034	0.022
<i>ATP1A2</i>	9.85	10.17	0.44	0.027	0.024
<i>ATP1B2</i>	9.41	9.83	0.44	0.056	0.029
<i>WIF1</i>	7.18	7.44	0.39	0.037	0.032
<i>AFF4</i>	7.66	7.80	0.17	0.022	0.033
<i>BAG3</i> <sup>e</sup>	7.74	8.25	0.45	0.035	0.033
<i>TUBB2B</i> <sup>e</sup>	12.31	12.66	0.32	0.029	0.033
<i>SDC4</i>	8.01	8.53	0.49	0.040	0.034
<i>CPT2</i>	7.43	7.59	0.15	0.029	0.035
<i>SLC39A12</i>	7.25	7.54	0.35	0.060	0.038
<i>PCDHB5</i>	6.65	6.69	0.05	0.073	0.040
<i>SLC1A2</i>	10.29	10.74	0.54	0.040	0.041
<i>GJA1</i>	8.78	9.23	0.54	0.054	0.047
<i>TMEM192</i>	6.71	6.75	0.04	0.050	0.048

<sup>a</sup>Mean gene expression levels in controls (or AUD cases) measured by Illumina's HT-12 v4 Gene Expression BeadChip.

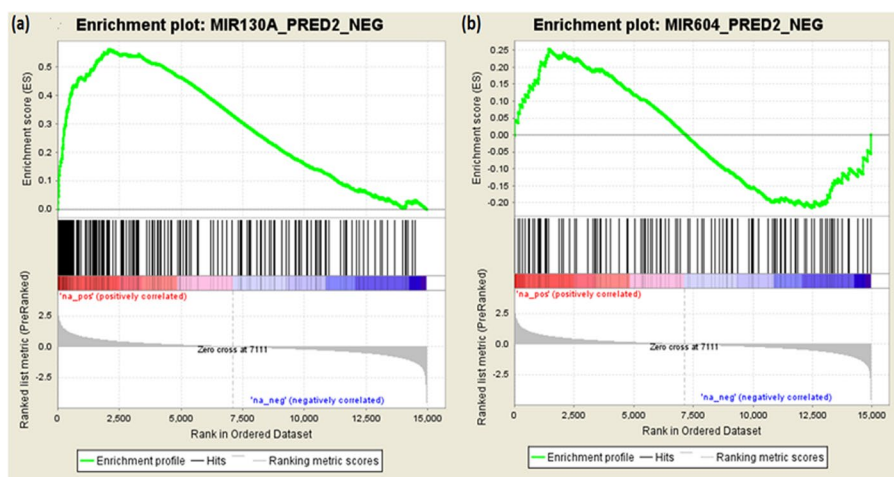
<sup>b</sup>Effect size (or regression coefficients) obtained by multiple linear regression analysis with adjustment for sex, age, and PMI.

<sup>c</sup>Observed  $P$  values obtained by multiple linear regression analysis with adjustment for sex, age, and PMI.

<sup>d</sup>Permutation  $P$  values obtained by R package lmpPerm analysis.

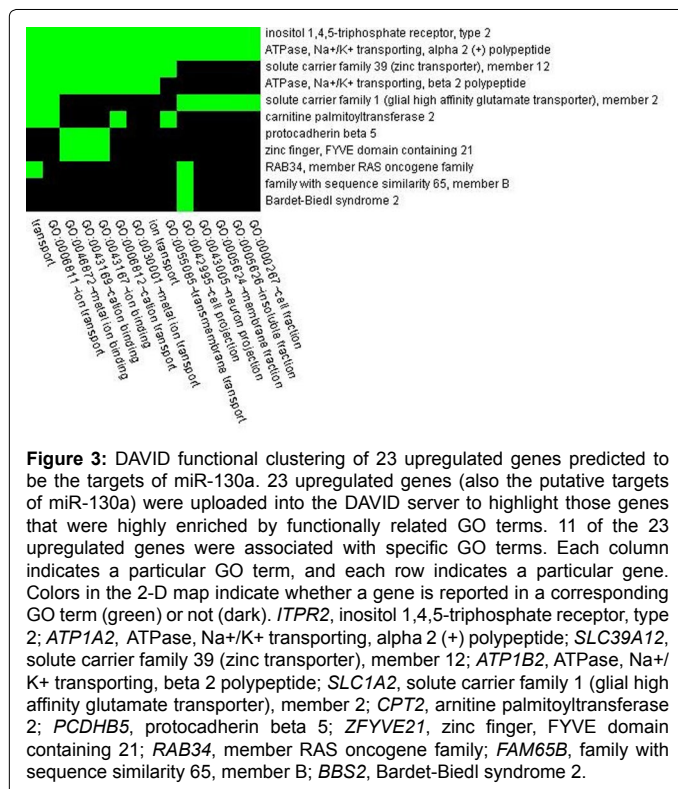
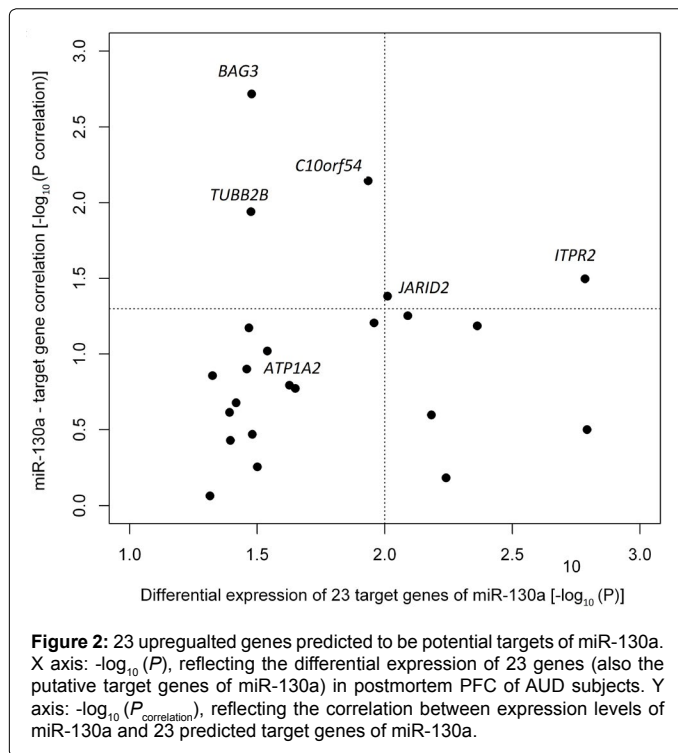
<sup>e</sup>Genes with expression levels negatively correlated with that of miR-130a.

**Table 1:** 23 differentially expressed genes predicted to be targets of miR-130a.



**Figure 1:** Gene set enrichment analysis of miR-130a and miR-604.

The enrichment scores (shown in the upper curve) were calculated along the ranked genes (the bottom histogram, from left to right, i.e., from upregulated to downregulated). The vertical lines in the middle indicate the location of the members of a given gene set. The maximum enrichment score not only reflects the possibility that a set of putative target genes of miR-130a is overrepresented by a set of the ranked genes, but also implies that the 77 leading genes made major contribution to the maximum enrichment score.



into the DAVID web server. Highly enriched gene ontology (GO) terms were assigned into eight clusters. As shown in Figure 3, the top cluster (with a fold enrichment score of 1.25) consisted of 14 GO terms, and 11 genes in this top cluster mainly participate in cell projection, ion transport, and neuron projection. Two genes (*ITPR2* and *ATP1A2*) were implicated in all 14 GO terms.

## Discussion

The chronic use of alcohol provokes gene expression changes, allowing the brain to adapt to the environment through homeostatic mechanisms in distinct signaling pathways. In the present study, genome-wide differential expression analysis revealed that two miRNAs (miR-130a and miR-604) and 787 coding genes were dysregulated in postmortem PFC of AUD subjects. Since miRNAs are able to inhibit the expression of their target mRNAs, the significantly negatively correlated miRNAs and mRNAs potentially constitute the functional miRNA-mRNA pairs. We used an integrative analysis approach to identify the putative target genes (or mRNAs) of miR-130a and miR-604. Gene set enrichment analysis indicated that the putative target genes of miR-130a (but not miR-604) were over-represented by differentially expressed genes.

Among the 23 upregulated genes that were predicted to be potential targets of miR-130a (Table 1), five (*ITPR2*, *JARID2*, *C10orf54*, *BAG3*, and *TUBB2B*) were negatively correlated with miR-130a in their expression levels. Two (*ITPR2* and *JARID2*) of them are of interest. Functional clustering analysis suggests that *ITPR2*, which is an ion channel gene, might be involved in the development of AUDs. *ITPR2* encodes the inositol 1, 4, 5-triphosphate receptor (type 2), which is a ligand-gated ion channel that can be activated by cytosolic Ca<sup>2+</sup> and inositol trisphosphate (IP3). A growing number of studies indicate that ethanol alters nerve signaling by interacting with neurotransmitter receptors and ion channels in the central nervous system, particularly the pentameric ligand-gated ion channels [e.g., GABA(A) and nicotinic acetylcholine receptors] [22] and voltage-gated calcium channels [23]. Genetic variants (rs1049380 and rs4654) in *ITPR2* have been reported to be associated with alcohol dependence by Genome-wide association studies [24]. Moreover, *ITPR2* and several other genes form a network to influence the function of neural synapses, and thus are potentially involved in traits such as substance use, stress, obesity and so on [25]. *JARID2* encodes a nuclear protein that is essential for embryogenesis, and overexpression of *JARID2* negatively regulates cell proliferation. Although the direct influence of *JARID2* on the risk of AUDs has not been observed, variation in *JARID2* has been found to be associated with AUD-relevant disorders such as schizophrenia [26,27]. To extend these findings, future studies are needed to examine whether the impact of miR-130a on the susceptibility of individuals to AUDs is through regulating the ion channel activity.

To our current knowledge, only one study conducted by Lewohl et al. [15] is known to have systematically examined AUD-associated miRNAs in postmortem brains of AUD subjects (14 AUD cases vs. 13 controls), and a total of 35 miRNAs were identified to be up-regulated in AUD cases. Compared to the study by Lewohl et al. [15], the novelty of the present study is that we used an integrative approach to screen the putative targets of miRNAs by combining miRNA target prediction and miRNA-mRNA expression correlation analysis. Nevertheless, the two AUD-associated miRNAs (miR-130a and miR-604) identified in the present study were not among the 35 miRNAs identified in the study by Lewohl et al. [15]. The inconsistent findings may be due to (1) the inclusion criteria of AUD cases were similar but may not be exact the same, thus leading to different genetic backgrounds; (2) the sample size of these two studies were different (the sample size was larger for the present study); and (3) both studies did not consider the accumulative effect of multiple miRNAs as well as the interactive effect of miRNAs and environmental factors on the risk for AUDs.

To have a better understanding of the role of miRNA-mRNA pairs in the etiology of AUDs, the present study needs to be improved in

several aspects. First, although the sample size is larger than that of any published studies that used postmortem PFC tissues for AUD studies, the moderate sample size (23 cases and 23 controls) may not have sufficient statistical power to identify most differentially expressed miRNAs and mRNAs. Thus, studies with a larger sample are warranted. Second, although we applied an integrative approach to identify putative targets of miRNAs, more efforts should be made to confirm the predicted target genes of miRNAs. Functional study approaches (e.g., 3' UTR-reporter gene assays) are necessary to validate the interaction of miRNAs and their target mRNAs. Third, the present study investigated the miRNA regulatory activity in only one brain region (PFC). The findings should be extended to other reward-related brain regions. Fourth, given the relatively low specificity and sensitivity of the microarray method, other advanced gene expression detection systems such as the next-generation sequencing technology (e.g., miRNA-Seq and mRNA-Seq) would more ideally be used.

In summary, the present study provided evidence that miR-130a and its target genes are involved in the neuroadaptations to alcohol. We thus conclude that dysfunction of miRNAs in human PFC may contribute to the etiological process of AUDs, and that miR-130a and its target genes are potential targets for pharmacotherapy of AUDs.

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