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Research paper

Profiling ethanol-targeted transcription factors in human carcinoma cell-derived embryoid bodies

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ABSTRACT

Fetal alcohol spectrum disorder is a collective term that represents fetal abnormalities associated with maternal alcohol consumption. Prenatal alcohol exposure and related anomalies are well characterized, but the molecular mechanism behind this phenomenon is not yet understood. Few insights have been gained from genetic and epigenetic studies of fetal alcohol spectrum disorder. Our aim was to profile the important molecular regulators of ethanol-related alterations of the genome. For this purpose, we have analyzed the gene expression pattern of human carcinoma cell-derived embryoid bodies in the absence or presence of ethanol. A cDNA microarray analysis was used to profile mRNA expression in embryoid bodies at day 7 with or without ethanol treatment. A total of 493 differentially expressed genes were identified in response to 50 mM ethanol exposure. Of these, 111 genes were up-regulated, and 382 were down-regulated. Gene ontology term enrichment analysis revealed that these genes are involved in important biological processes: neurological system processes, cognition, behavior, sensory perception of smell, taste and chemical stimuli and synaptic transmission. Similarly, the enrichment of disease-related genes included relevant categories such as neurological diseases, developmental disorders, skeletal and muscular disorders, and connective tissue disorders. Furthermore, we have identified a group of 26 genes that encode transcription factors. We validated the relative gene expression of several transcription factors using quantitative real time PCR. We hope that our study substantially contributes to the understanding of the molecular mechanisms underlying the pathology of alcohol-mediated anomalies and facilitates further research.

1. Introduction

Drinking alcoholic beverages is a common cultural practice among humans, even though it is well characterized as a classic teratogen. Previous reports showed that maternal drinking causes abnormalities in the developing fetus (Roszel, 2015; Halder et al., 2014; Lewis et al., 2015). The most common abnormalities are craniofacial, growth, central nervous system (CNS), and neurobehavioral abnormalities. Associated psychosocial problems include learning difficulties, attention deficit–hyperactivity disorder (ADHD), and mental retardation (Burd et al., 2003; O'Leary, 2004). Although extensive research has been performed to further our understanding of the mechanisms of ethanol (EtOH)-induced malformations, the molecular pathway(s) leading to fetal alcohol syndrome (FAS) are still unknown.

Over the past decades, tremendous progress has been achieved in the research area related to alcoholic toxicity during fetal development. Among researchers in the field of chronic, acute and binge alcohol exposure, research related to genomic and epigenomic alteration is of particular interest. Previous studies in this area showed that distinct pathways are involved in alcohol-related toxicity. For example, several signaling pathways were found to exert EtOH mediated actions, including those related to, retinoic acid (Halder et al., 2014; Kerns et al., 2005), glucocorticoids (Kerns et al., 2005; Bell et al., 2009), the stress response (Saito et al., 2002; Treadwell and Singh, 2004), the mitogen-activated protein kinase (MAPK) (Arlinde et al., 2004), neurotransmitters (Hu et al., 2008; Ponomarev et al., 2012), phosphoinositide 3-kinase (Daniels and Buck, 2002), calcium (Covarrubias et al., 2005), Wnt signaling (Vangipuram and Lyman, 2012; Mandal et al., 2015), and Notch and JAK/STAT (Daniels and Buck, 2002).

Transcription factors (TFs) are responsible for the specific gene expression patterns necessary for a cell to perform its unique functions

Abbreviations: NCCIT, human embryonic carcinoma cell; EB, embryoid body; EtOH, ethanol; TFs, transcription factors; FASD, fetal alcohol spectrum disorder; GO, gene ontology; DEG, differentially expressed gene.

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(Ebina and Rossi, 2015). Mechanistically, TFs can change the chromatin state by directly recruiting modifiers to the specific motifs found in gene regulatory regions such as promoters and enhancers (Rosenfeld et al., 2006). The recruitment of transcriptional coactivators or corepressors by a particular TF determines whether gene expression is promoted or suppressed, respectively. Additionally, a TF can enforce a particular cell fate by stimulating genes required for progressive differentiation and the suppression of lineage inappropriate genes (Pongubala et al., 2008; Schaffer et al., 2010). Alteration of these precise expression patterns in the cell can cause abnormal differentiation as well as defective cellular functions. Alcohol-mediated alteration of TFs is not well studied and requires more attention.

In the present study, we have set up an in vitro model for chronic EtOH exposure on human embryonic carcinoma (NCCIT) cell-derived embryoid bodies (EBs) that mimic the early development of the fetus. Our ultimate goal was to profile novel genes that were altered by EtOH to facilitate future research of alcohol-related fetal abnormalities. We compare gene expression profiles in the presence and absence of EtOH by microarray analysis. We also carried out extensive bioinformatics analysis on the gene expression data and identified a group of genes that encode TFs during embryonic development.

2. Materials and methods

2.1. Cell culture and EtOH treatment

We used NCCIT cells that were obtained from the American Type Culture Collection (CRL-2073). The cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 IU ml–1 penicillin and 10 μg ml–1 streptomycin at 37 °C in the CO_2 incubator. For EB formation, 1×10^6 cells were plated in absence or presence of EtOH (50 mM) in 90-mm bacterial culture dishes (non-adherent culture conditions) for 7 days. We changed the culture media daily to maintain the treated EtOH concentration during the course of the study. To prevent EtOH evaporation from the culture dishes, EtOH-treated cells were cultured in a separate CO_2 incubator that was saturated with 50 mM EtOH, as described previously by our group (Halder et al., 2015).

2.2. Microarray analysis

Total RNA (~8 μg) was isolated for different samples using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA quality was assessed using the Agilent 2100 Bioanalyzer with the RNA 6000 Nano Chip (Agilent Technologies, Waldbronn, Germany), and the quantity was determined using a spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Transcriptomic analysis was performed using the Affymetrix GeneChip® Human Gene 1.0 ST Array. The sample preparation, labeling and hybridization were performed according to the manufacturer's instructions and recommendations. Image acquisition was performed with the Affymetrix GeneChip® Scanner 3000 7G. The imaging data were analyzed with the GeneChip® Operating Software (GCOS) using the Affymetrix default analysis settings. The data set supporting the result of this article is available in the NCBI's Gene Expression Omnibus (GEO) repository under the accession number GSM1657345 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1657345).

2.3. Gene ontology (GO) analysis

Functional annotation of significant genes identified by the microarray analysis was determined using the web accessible program, Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov). DAVID calculates a modified Fisher's exact p-value to demonstrate GO, where p-values less than 0.05 are considered strongly enriched in the annotation category.

2.4. Construction of heat maps

We constructed heat maps to view the relative expression patters of our array data using the TIGR Multiexperiment Viewer (MeV), which is a Java-based microarray data analysis tool (desktop application) that allows advanced analysis of gene expression data through an intuitive graphical user interface. We uploaded our array data in text file format and chose two color arrays to create heat maps.

2.5. Enrichment analysis for diseases

Integrated disease enrichment analysis was performed using ingenuity pathway analysis (IPA) version 21901358. The differentially expressed genes (DEGs) were mapped into the analysis tool for observation of the significant disease pathway enrichment. This analysis helped us to understand causal connections between diseases and genes.

2.6. Quantitative reverse transcription-polymerase chain reaction

To measure gene expression, we performed real-time (RT)-PCR using the ABI 7500 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). Total mRNA was reverse transcribed into cDNA using PrimeScript TM Reverse Transcriptase (Takara Bio Inc., Otsu, Shiga, Japan). The final volume of the PCR mixture was 20 μl, and the threshold cycle (Ct) values were normalized to the C_t for Gapdh mRNA. The gene expression patterns by growth phase at 37 °C or at different temperatures were calculated using the $2^{-\Delta\Delta CT}$ method (Halder et al., 2015). The primers employed for amplification are listed in Supplemental Table 1.

2.7. Statistical analysis

In this study, we investigated the relative gene expression between control and experimental samples using a cDNA microarray analysis. For the qRT-PCR analysis we set three separate experiments and results are presented as the mean ± standard error of the mean (SEM). The transcription factors were randomly chosen from the list for qRT-PCR analysis. For the statistical analyses, Student's t-test was performed using the Microsoft Office Excel, 2010 program at the 0.05 probability level.

3. Results

3.1. Genes are differentially expressed in EtOH-treated EBs

We cultured EBs for 7 days in presence or absence of EtOH to observe the effect of EtOH on EB formation. To explore the effects on a genomic level, we performed a microarray analysis for EB 7D vs. EB + EtOH 7D using the Affymetrix GeneChip® Human Gene 1.0 ST Array. We observed that genes were differentially expressed in EtOH-treated EBs compared to controls. We recorded altered expression of 493 genes at a 1.5-fold cut-off value; the numbers of up- and down-regulated genes were 111 and 382, respectively. Remarkably, we noted that the EtOH-induced suppression of gene expression was more than three times higher than that of induction. The number of altered genes showed us a clear view of the deleterious effect of EtOH during early development. Based on the fold-change criteria above used to mine the biological data, a heat map was constructed showing that the expression of genes in the EtOH-treated EBs was clearly different from that in the controls (Fig. 1a).