Systems Analysis of Ethanol Metabolism and Voluntary Alcohol Consumption: Using the HRDP

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Abstract

We have collected whole transcriptome data for several organs of a subset (30) of the Hybrid Rat Diversity Panel (HRDP) and have used quantitative genetics and Weighted Gene Co-expression Analysis (WGCNA) of brain and liver transcriptomes to investigate the predisposing factors for the variation in voluntary alcohol consumption. We found two QTLs for the trait of alcohol consumption and our criteria for linking the transcriptome data to the behavioral data was based on the correlation of the eigengene values for the co-expression modules with the behavior and the overlap of the genomic location of the behavioral QTL with the QTL for the co-expression module eigengene. When examining the brain data, we found a module that satisfied all criteria and the eigengene QTL overlapped the behavioral QTL on Ch. 12. The hub gene for this module was an lncRNA and we demonstrated a functional relationship of this lncRNA and alcohol consumption. However, we did not find any brain co-expression modules related to the behavioral QTL on Ch. 1. When we examined the co-expression modules derived from the liver transcriptome we identified a module which met all criteria and overlapped the alcohol consumption QTL on Ch. 1. This module explained 24% of the genetic variance in alcohol consumption. The transcripts included in the liver module brought attention to liver products influencing immune and inflammatory pathways and give credence to the hypothesis relating inflammatory systems in brain and periphery to voluntary alcohol consumption. We also used out unsupervised statistical approach to explore the factors contributing to differential metabolism of ethanol across 30 RI strains. We identified a co-expression module from the liver transcriptome whose mQTL overlapped the phenotypic QTL for the rate of ethanol disappearance and, as expected, contained the alcohol dehydrogenase gene transcripts known to be primarily responsible for ethanol metabolism in the rat and human.
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Background

The full HRDP when completed will be a combination of recombinant inbred (HXB/BXH and FXLE/LEXF) and classic inbred strains of rats that optimizes genetic mapping power and precision. It will provide a genetically stable and diverse population lending itself to multi-omics studies. As can be seen below the available strains and data available currently are about half of the final 96 strain two tissue HRDP dataset which includes extensive sequencing of the transcriptome. The PhenoGen website (https://phenogen.org) provides data and tools to apply both phenotype-driven analyses and gene-driven analyses to understand the contribution that a genetic network can make to a phenotype. The following results represent two examples where the current data set has been used with a systems genetic methodology in relation to alcohol consumption and metabolism. First, we previously used an integrative genomics approach to identify transcriptional networks in rat brain that are associated with a predisposition to consume varying amounts of alcohol (Saba et al, 2015). The method outlined below narrowed our focus to one module (Figure 1A) that met all criteria for assigning a functional relationship between the members of this module and the predisposition to differences in alcohol drinking levels among strains. The module hub gene (most highly connected gene) is an unannotated transcript, whose sequence suggests that it is likely to be a long intergenic non-coding (linc)RNA. Its expression level is negatively correlated with alcohol consumption in the HXB/BXH RI panel (Figure 1B). To ascertain the role of this transcript in modulating alcohol consumption, we first statistically analyzed its influence on the candidate co-expression module (Figure 2), and then used CRISPR/Cas9 technology to remove the third exon of this transcript in Wistar rats, and assessed voluntary alcohol consumption by the genetically modified rats (Figures 3 and 4).

The second example was designed as a proof-of-concept experiment, using liver transcriptome data to ascertain module(s) linked to ethanol clearance and circulating acetate levels using this analysis pipeline. Rats from the HXB/BXH RI panel were injected with 2g/kg of alcohol and circulating levels of alcohol and acetate were measured over time(Lusk et al, 2018). Using the method described below a single candidate module for these phenotypes was identified.

Transcriptome Data Collected (Exon Arrays and RNA-Seq on total RNA) Strains Already Completed for Brain, Liver, Heart

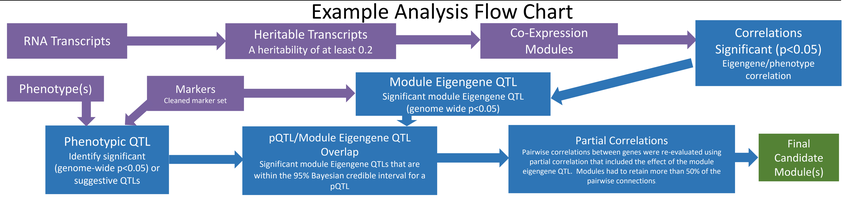
HXB1	HXB21	BXH6	LEW/SsNHsd
HXB2	HXB22	BXH8	SHRSP
HXB3	HXB23	BXH9	WKY/NCrl
HXB4	HXB24	BXH10	ACI/SegHsd
HXB5	HXB25	BXH11	Dark Agouti
HXB7	HXB26	BXH12	SS/JrHsd
HXB10	HXB27	BXH13	F344/NHsd
HXB13	HXB29	BN-Lx/Cub	F344/Stm
HXB15	HXB31	SHR/Ola	LE/Stm
HXB17	BXH2	SR/JrHsd	
HXB18	BXH3	COP/CRL	
HXB20	BXH5	F344/NCr	

Summary of RNA-Seq Data Collected

Strains	Tissue	Sex	Number of Biological Replicates Per Strain	Number of Paired-End Reads (rRNA-depleted total RNA)	Number of Single-End Reads (small RNA)
SHR and BNLx	Brain	male	3	645 million	96 million
SHR and BNLx	Brain	female	4	982 million	297 million
SHR and BNLx	Liver	male	3	583 million	342 million
SHR and BNLx	Heart	male	4	790 million	300 million
45 HRDP Strains	Brain	male	4(RI) 3(Inbred)	17.0 billion	5.0 billion
45 HRDP Strains	Liver	male	4 (3-UCD 1-Euratrans)	15.8 billion	5.1 billion
30 HRDP Strains	Heart	male	1(RI) 5(Parentals)	5.5 billion	-

Methods

Example Analysis Flow Chart



Influence of Candidate Module Eigengene and Hub Gene on Module Characteristics: To explore potential drivers of co-expression within the candidate brain co-expression module for alcohol consumption shown in Figure 1, we compared the correlations between transcripts with partial correlations which account for the module eigengene QTL or LRAP expression (Figure 2). In order to compare the magnitude of these correlations, we first re-visualized the candidate co-expression module using correlation coefficients rather the connectivity, as defined in the original network graphics (Figure 1, Saba et al., 2015). Pairwise correlations between RNA expression levels of genes within the candidate co-expression module were determined using Pearson correlation. To determine if the correlation between any two genes from the candidate co-expression module was maintained after accounting for variation in expression due to the module eigengene QTL or due to the expression of LRAP, we calculated partial correlation coefficients using the *ppcor* package in R (version 3.5.0). Network figures were generated using Cytoscape (version 3.6.1).

Creation of genetically modified rats: CRISPR/Cas9 gene targeting was used to disrupt the third exon of the LRAP gene (hub gene of the co-expression module associated with alcohol consumption, see Figure 1) in Wistar rats (Hsu et al., 2013; Cong et al., 2013). These rats were generated by Dr. Gregg Homanics, University of Pittsburgh.

qRT-PCR analysis: Knockout of the third exon of the hub gene was assessed in rat brain using qRT-PCR. Designs for primers that assay the LRAP sequence were limited to specific regions of sequence unique to each of three exons. Primers were selected using Primer3 (http://frodo.wi.mit.edu/) (Saba et al., 2015).

Voluntary Alcohol Consumption, QTL Analysis, and gene co-expression network analysis (WGCNA) as previously described (Saba et al, 2015).

Alcohol Clearance and Acetate Levels were measured as previously described (Lusk et al, 2018)

Results

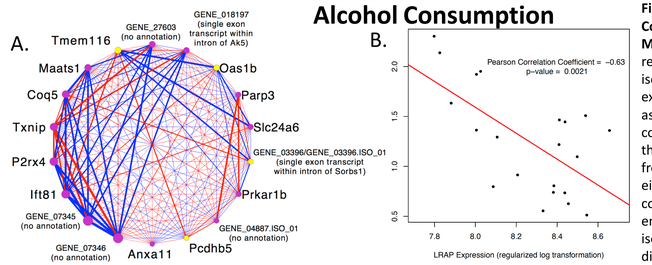


Figure 1: LincRNA Alcohol Consumption Candidate Module. A. Each node represents a gene and/or an isoform from two co-expression modules that were associated with alcohol consumption using a p-value that combined information from the correlation of the eigengene with alcohol consumption and the enrichment of genes / isoforms within modules differentially expressed in rat lines selectively bred for high or low alcohol consumption(Saba et al, 2015). The size of the node is weighted based on its intramodular connectivity. Nodes highlighted in yellow represent genes identified in both the gene-level analysis and the isoform-level analysis. The thickness of the line connecting two nodes, i.e., edge, is weighted based on the magnitude of the correlation coefficient between the two genes. Red edges represent a negative correlation and blue edges represent a positive correlation. **B.** Negative correlation between hub gene, Locus Regulating Alcohol Preference (LRAP), expression in brains of alcohol-naïve male rats from the HXB/BXH recombinant inbred panel and average daily alcohol consumption from the 2nd week of the two-bottle choice paradigm. Expression levels were measured by RNA-Seq (confirmation of array results) and 1-3 biological replicates per strain. Circles represent strain means for both alcohol consumption and RNA expression.

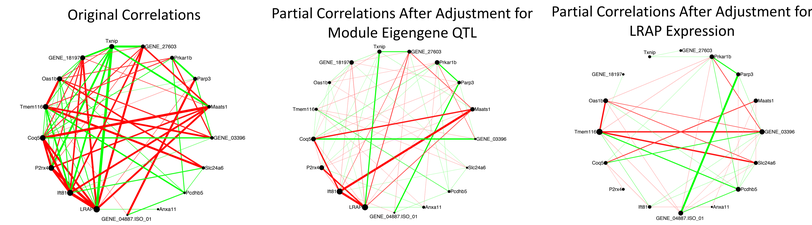


Figure 2. Comparison of within module correlations after adjustment for possible sources of co-regulation. Circles represent genes from the original candidate brain co-expression module related to alcohol consumption shown in Figure 1. However, in this figure, the size of the each circle is related to the gene's intramodular correlation. A line connecting two genes indicates that the correlation or partial correlation between the two genes is greater than 0.3 or less than -0.3. A green line indicates a negative correlation and a red line indicates a positive correlation. The thickness of the line represents the magnitude of the represented correlation.

Generation of Genetically Modified Rats Many more correlations were maintained after accounting for the influence of the module eigengene QTL than after accounting for the influence of the hub gene (LRAP), i.e., the number of connections in the module was dramatically reduced by eliminating the influence of the hub gene on expression levels of the other genes. These results suggest that the majority of the correlations within the candidate module are due to shared control of expression by LRAP. To determine the effect on the phenotype of alcohol consumption of disruption of this key influence that led to loss of candidate module structure, we generated a physical disruption of LRAP using CRISPR/Cas9 methodology.

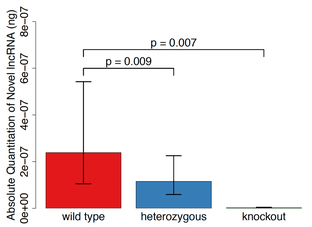


Figure 3: Expression differences in LRAP between genetically modified rats. qRT-PCR results for expression levels of exons 1-3 in rat brains are reported as absolute quantity (n= 3/genotype). This PCR product is undetectable in the KO rats.

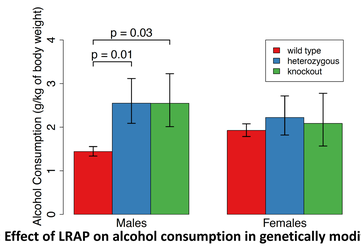


Figure 4: Effect of LRAP on alcohol consumption in genetically modified rats. Alcohol consumption in the two bottle choice paradigm was measured in the 3 different genotypes. The means and standard errors reported are for the average daily ethanol consumption during the second week. The two horizontal lines represent pairwise comparisons that reached statistical significance (p<0.05). Differential consumption was calculated using a 2-way analysis of variance with a heterogeneous covariance structure to account for the dramatically different variances within genotype and batch. Alcohol consumption measures were transformed using a log base 2 prior to analysis.

Alcohol Clearance and Circulating Acetate

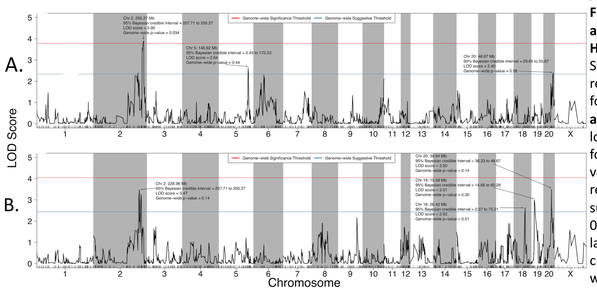
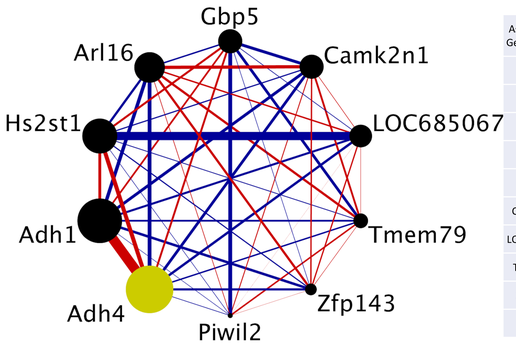


Figure 5: Quantitative trait loci for alcohol clearance and acetate AUC in the HXB/BXH recombinant inbred panel. Strain means were used in a marker regression to determine phenotypic QTL for **(5a) alcohol clearance** and **(5b) acetate AUC.** The red lines represent the logarithm of odds (LOD) score threshold for a significant QTL (genome-wide p-value = 0.05), and the blue lines represent the LOD threshold for a suggestive QTL (genome-wide p-value = 0.63). Significant and suggestive QTL are labeled with their location, 95% Bayesian credible interval, LOD score, and genome-wide p-value. (Lusk et al., 2018)



Associated Gene Name	Type	Description	Intra-modular Connectivity
Adh4	Protein coding	Alcohol dehydrogenase 4 (class II)	1.18
Adh1	Protein coding	Alcohol dehydrogenase 1 (class I)	1.17
Hs2st1	Protein coding	Heparan sulfate 2-O-sulfotransferase 1	1.14
Arl16	Protein coding	ADP-ribosylation factor-like 16	1.13
Gbp5	Protein coding	Guanylate binding protein 5	1.11
Camk2n1	Protein coding	Calcium/calmodulin-dependent protein kinase II inhibitor 1	1.11
LOC685067	Protein coding	Similar to guanylate binding protein family, member 6	1.10
Tmem79	Protein coding	Transmembrane protein 79	1.08
Zfp143	Protein coding	Zinc finger protein 143	1.07
Piwil2	Protein coding	Piwi-like RNA-mediated gene silencing 2	1.05

Figure 6: Connectivity within the candidate co-expression module for both alcohol clearance and acetate AUC. Each circle represents a gene from the co-expression module. The size of each circle is weighted based on its intra-modular connectivity (not to scale), and the thickness of each edge is weighted based on the magnitude of the connectivity between the two genes (not to scale). The edge colors indicate the direction of the connectivity (red = positive, blue = negative). The hub gene, defined here as the single gene with the largest intra-modular connectivity, is colored in yellow (*Adh4* = alcohol dehydrogenase 4), and its expression is positively associated with both alcohol clearance and acetate AUC. The figure was generated using Cytoscape (v. 3.4.0)(Lusk et al., 2018)

Discussion

The integrative genomic approach that we have previously described as a method to identify candidate genes and networks that predispose to differences in alcohol consumption (Saba et al., 2015; Tabakoff et al., 2009) utilizes a pipeline that we have developed to integrate information on complex behavioral/physiological traits and gene expression (RNA-Seq, microarray) data. This pipeline uses recombinant inbred and/or inbred animals, and involves: 1) obtaining behavioral/physiological QTLs; 2) obtaining tissue-specific gene expression and co-expression modules for the organ or tissue involved in the trait of interest; 3) ascertaining the correlation of module eigengene values and phenotypic trait values across strains; 4) for correlated modules, determining whether the eigengene QTL overlaps the phenotypic QTL. This approach, using male rats, led us to the transcriptional module shown in Figure 1A and 2.

The level of expression of the hub gene in brains of the males of the HXB/BXH RI rat strains was negatively correlated with alcohol consumption. We determined by partial correlation analysis that elimination of the influence of the hub gene on gene expression caused a dramatic disruption of candidate module structure. In order to validate these results in an in vivo system, we created rats in which the hub gene (LRAP) for this module was disrupted. This gene is an unannotated transcript, and examination of the sequence of the gene, including the large number of stop codons, suggests that it may be a long intergenic non-coding RNA. Disrupting the gene led to a significant increase in alcohol consumption by male rats, in which the module was identified. These results represent the first demonstration that a putative lincRNA can modulate alcohol consumption, and validate the approach used to identify candidate genes/networks that influence the predisposition for this complex trait. The qRT-PCR results measuring the expression of the LRAP gene proved surprising. Little to no RNA coding for LRAP was expressed in either the male HET or KO rats. Correspondingly, the increase in alcohol consumption was equivalent in the HET and KO rats compared to the male WT rats. However, there was no difference among female WT, HET and KO rats, and we are currently investigating potential differences between males and females with respect to the actions of LRAP.

As a further proof of concept, we applied our approach to the phenotypes of alcohol clearance, and circulating acetate levels after alcohol administration, across the HXB/BXH panel. One module met the criteria for correlation with phenotype and overlapping QTLs. It contains two alcohol dehydrogenase transcripts that are mainly expressed in liver, consistent with expectations. However, in addition it provides links to other interesting pathways with additional functionality of ADH enzymes in retinol metabolism, and Arl16, both with links to RIG-I involved in antiviral immunity(Matsumiya and Stafforini, 2010). Hs2st1 and Camk2n1 are also both related to regulation of aspects of the immune system (Xu et al., 2007, Khanna et al., 2017, Kim et al., 2017, Kreuger et al., 2006). Zfp143 is linked to regulation by linking promoter regions to distant regulatory elements (Bailey et al., 2015). This approach has shown promise in identifying novel regulatory genes related to the phenotype studied and demonstrated that expected results as well as other interactions worth pursuing can be obtained by using the HRDP data with relevant phenotype data.

Conclusion

The information provided on PhenoGen (https://phenogen.org) can be used without additional data, or with a phenotype assessment of a trait using the HRDP rats. The PhenoGen data reflect a basal state of the animal and should be considered to reflect a predisposition to a complex trait. The analysis of quantitative phenotypes by the researcher and the use of data provided by PhenoGen can be used 1) to explore the factors predisposing to disease or the normal variance in measured phenotypes, 2) predict differences in response to therapy, 3) Identify novel therapeutic targets. If you can model an addiction or any other phenotype in a rat you can deduce the predisposing genetic factors for that phenotype.

Using these methods we found an unannotated gene that was the hub gene of a network related to alcohol consumption. As validation of the influence of this module hub gene, part of the gene was knocked out and alcohol consumption measured on these animals. The resulting males showed a significant increase in alcohol consumption while interestingly females did not. However the network containing this hub gene was generated based on data from only male rats. Further proof of concept for this systems genetic approach is the analysis of the alcohol clearance phenotype where blood alcohol and acetate levels in blood were measured over time. The analysis method produced a single candidate module primarily containing genes known to be responsible for alcohol metabolism, expressed in liver, and containing a link to the immune system. This method demonstrates the overall accuracy of this approach and the usefulness of the HRDP resource, while also highlighting that careful inspection of the results is required when drawing conclusions from this approach.

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