Altered Gamma-Aminobutyric Acid Type B Receptor Subunit 1 Splicing In Alcoholics

Changhoon Lee, R. Dayne Mayfield, and R. Adron Harris

Background: Chronic alcohol exposure can change splice variant expression. The gamma-aminobutyric acid type B (GABAB) receptor undergoes splicing and is an alcoholism treatment target, but there is little information about splicing changes in this receptor in alcoholics. We studied GABAB receptor subunit 1 (GABAB1) splicing in alcoholic postmortem brains.

Methods: To maximize GABAB1 splice junction identification, we combined gene specific libraries with RNA-seq. Splice junctions and mapped reads were also found from intronic and intergenic regions. We compared GABAB1 splice junctions in prefrontal cortices from 14 alcoholic and 15 control subjects and introduced new strategies, reads per kilobase of splice junction model per million mapped reads and reads per kilobase of gene model per million mapped reads, for quantitating splice junction and gene expression.

Results: Novel splice junction detection indicated that the *GABAB1* gene is at least two times longer than the previously reported gene length. GABAB1 exon and intron expression data showed low expression at the 5′ end exons and exon grouping. This indicated that there are short splicing variants in addition to GABAB receptor subunit GABAB1a, the longest known major transcript. We found that chronic alcohol altered exon/intron expression and splice junction levels. Decreased expression of the gamma-aminobutyric acid binding site, a transmembrane domain and a microRNA binding site may decrease normal GABAB1 transcript population and thereby decrease normal signal transduction in alcoholics.

Conclusions: We discovered novel, complex splicing of GABAB1 in human brain and showed that chronic alcohol produces additional splicing complexity.

Key Words: Alcoholism, exon/intron expression, GABAB receptor, human prefrontal cortex, RNA-Seq, splice junction

Gamma-aminobutyric acid type B (GABAB) receptors have been implicated in regulation of alcohol drinking. The positive allosteric modulator, GS39783, suppresses alcohol drinking and reinforcement in rats (1). A GABAB receptor agonist, gamma-hydroxybutyrate, can reduce voluntary ethanol drinking and withdrawal symptoms in humans and is a treatment for alcoholism (2,3). Another agonist, baclofen, may also be effective in treating the disease (4).

Previous microarray studies showed strong GABAB receptor subunit 1 (GABAB1) expression in human alcoholic prefrontal cortices (5,6). However, these microarray experiments used complementary DNA (cDNA) probes, and two out of three GABAB1 probes were complementary to regions apparently generated by unknown splicings. For example, clone 300899 was aligned to GABAB1 intron 4. It is a common intron region from the RefSeq Genes model and major GABAB1 splicing variant (7). Five different splicing variants were cloned containing clone 300899 (7,8) including GABAB receptor subunit GABAB1m spliced out at exon 6 (7). Another probe, clone 2312175, targeted an unknown splicing out at exon 23. Thus, the microarray experiments and splice variants suggested complex *GABAB1* gene splicing in human brain (7–10).

From the Waggoner Center for Alcohol and Addiction Research, Section of Neurobiology and Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas.

Address correspondence to Changhoon Lee, Ph.D., The University of Texas at Austin, Waggoner Center for Alcohol and Addiction Research, Section of Neurobiology and Institute for Cellular and Molecular Biology, 2500 Speedway, MBB 1.124, Austin, TX 78712; E-mail: changhoonlee@ucla.edu.

Received Nov 27, 2012; revised and accepted Aug 27, 2013.

Among the three microarray probes, only clone 300899 showed increased expression in alcoholics (5,6). This suggested a specific GABAB1 splicing change is related with chronic alcohol exposure. Signal intensities of the three probes were different, and the difference was not explicable based on known splicing variants. This suggested that unknown GABAB1 splicing variants are differentially expressed in alcoholic brain. Single nucleotide polymorphisms near splicing sites also alter splicing and may be associated with alcoholism (11–13). Chronic ethanol exposure and withdrawal increased 5′ splice variant expression of the *N*-methyl-D-aspartic acid receptor subunit, NR1, without NR1 3′ variant expression changes (14). The L-type voltage-gated calcium channel, $\alpha1_c$, has two splice variants, $\alpha1_{c\text{-}1}$ and $\alpha1_{c\text{-}2}$; chronic ethanol treatment enhanced splicing resulting in a specific increase in the $\alpha1_{c\text{-}1}$ population (15). Other studies have also shown that splicing changes in brain can contribute to a wide range of neuropsychiatric disorders (11).

One approach to identifying novel splice junctions of GABAB1 is to query the National Center for Biotechnology Information database. However, this database may only contain a small fraction of the actual splice variants (16). We propose that many unknown splicings of the *GABAB1* gene may exist and propose use of RNA-Seq to detect new GABAB1 variants and to quantitate splicing differences in alcoholic brain.

Methods and Materials

RNA Sample Collection

Commercial human prefrontal cortex RNA was obtained from Ambion (Austin, Texas). Fifteen control and 14 alcoholic postmortem prefrontal cortices were obtained from the Tissue Resource Centre at the University of Sydney. The samples were matched as closely as possible by age, gender, brain pH, RNA integrity number, and postmortem interval (Table S1 in Supplement 1). Control subjects were defined as those who drank <20 g/day (most were social drinkers or teetotallers). Alcoholics

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2013.08.028

were defined based on alcohol consumption of >80 g/day. Diagnoses were confirmed by physician interviews, hospital medical record reviews, questionnaires to next-of-kin, and from pathology, radiology, and neuropsychology reports. The DSM-IV diagnosis was based on a clinical assessment by specially trained staff in psychiatry or psychology (17). After RNA extraction DNase (Ambion, Austin, Texas) was treated, and ribosomal RNA (rRNA) was depleted using the RiboMinus kit (Invitrogen, Carlsbad, California).

RNA-Seq Library Construction and Sequencing

For gene specific library, gene specific primer (GB1-Cter-1st-4, 5′-TCCCAGAGGTATGAG-3′) was optimized for reverse transcription at 42°C using SMARTer polymerase chain reaction (PCR) cDNA Synthesis Kit (Clontech, Mountain View, California). Using a template switching method (18), double strand cDNAs were generated from the commercial prefrontal cortex RNAs. For the amplification step, SMARTer2A-22mer (5′-AAGCAGTGGTATCAACGCAGAG-3′) was designed based on a provided primer, SMARTerIIA Oligonucleotide, which was bound at the 5′ end of transcripts during the template switching step. After amplification with SMARTer2A-22mer and another gene specific primer (GB1-Cter-2nd-1, 5′-CTACTGGCCTGTCCTCCCTCA-3′), double strand cDNA generation was confirmed. Gel extraction removed unbound primers and small amplification products. Following the SOLiD 3 System Library Preparation Guide (ABI, Carlsbad, California), gene specific library was prepared. For libraries of alcoholic and control samples, we used previously described primer sets (19).

Whole transcriptome libraries were prepared using total RNAs from 15 control and 14 alcoholic postmortem prefrontal cortices and commercial RNAs following the SOLiD Total RNA-Seq Kit (ABI, Carlsbad, California). The rRNA depleted RNAs were fragmented, and adaptors were ligated to their ends; cDNAs were then synthesized. The gene specific and whole transcriptome libraries were sequenced using the ABI SOLiD system.

Data Analysis for RNA-Seq Data

Total raw reads were filtered by sequencing quality. For every read, the number of quality values that were below 8 were counted. The reads were removed if the numbers were over 9 or 14 for 35mer or 50mer single end reads, respectively. Noncoding RNAs (mostly rRNAs and transfer RNAs) and adaptors were filtered out using Mapreads mapper (ABI). Two mismatches out of 20mer reads were allowed for the mapper. The filtered sequencing data were modified to .fq files using fq_all2std.pl program (http://maq.sourceforge.net/fq_all2std.pl), removing reads that contained quality values of −1. For each sample, TopHat mapping was performed against the human reference genome (hg18) using default options (20). To visualize the mapping data, .sam files were modified using igvtools (http://www.broadinstitute.org/software/igv/download) and visualized using the Integrative Genomics Viewer (http://www.broadinstitute.org/software/igv/) including another TopHat output (.wig file).

To maximize splice junction mining from whole transcriptome libraries, a splice junction search step was implemented before final mapping. At this step, we collected splice junctions from the .bed files of the individual mapping results from all gene specific and whole transcriptome libraries. After the combined .bed files were converted uniquely to a .juncs file, the alcoholic and control whole transcriptome library data were remapped using the .juncs file with −j option of TopHat. After recollecting splice junction data from the mapping, we generated a new .juncs file that contained additional splice junction information and visualized it using the University of California, Santa Cruz (UCSC) genome browser.

Expression Analysis Using Reads per Kilobase of Gene Model per Million Mapped Reads

Using supercomputers at the Texas Advanced Computing Center, reads per kilobase of gene model per million mapped reads (RPGM) values were calculated for genes, exons, and introns based on the formula below:

$$RPGM = \frac{mapped\ read\ \#}{\frac{total\ mapped\ read\ \#}{1{,}000{,}000} \times \frac{gene\ length\ (bp)}{1{,}000}}$$

Uniquely mapped reads were counted for each gene from whole transcriptome libraries of alcoholic and control samples. The mapped reads were normalized per million unique mapped reads of the whole human reference genome per kilobase of gene length.

RPGMs were calculated for the three different *GABAB1* gene lengths. After $\log_2$ transformation of the RPGM values, we used two-tailed unpaired *t* tests. We also calculated genome wide RPGMs for all genes, transcripts, exons, and introns based on RefSeq Genes model. The RPGM value differences between alcoholic and control groups were tested using DEGseq (21). *GABAB1* specific data were then selected from the analysis.

Splice Junction Analysis in Alcoholic Samples

To study GABAB1 splice junction changes in alcoholic brains, we calculated reads per kilobase of splice junction model per million mapped reads (RPJM) values for all splice junctions. The formula below was used for the RPJM calculation:

$$RPJM = \frac{mapped\ read\ \#}{\frac{total\ mapped\ read\ \#}{1{,}000{,}000} \times \frac{splice\ junction\ area\ length\ (bp)}{1{,}000}}$$

Uniquely mapped reads were counted for each splice junction from alcoholic and control whole transcriptome libraries. The mapped reads were normalized per total mapped read number per kilobase of splice junction area length where mapped reads can reach the splice junction. The splice junction area length is (read length −1) × 2.

After calculating genome wide RPJMs, RPJM value differences between alcoholic and control groups were also analyzed using DEGseq. GABAB1 splice junctions that significantly change in alcoholic brains were selected only if they were found in multiple samples.

Results

Gene Specific Library Construction to Maximize GABAB1 Splice Junction Detection

Gene specific library was designed for RNA-Seq to maximize splice junction identification in the *GABAB1* gene. Current approaches to RNA-Seq library construction often utilize random primers or oligo(dT) primers for reverse transcription (22–26). However, these approaches do not generate enough mapped reads to find rare splice junctions from a single RNA-Seq run. To provide a large population of gene specific reads, we constructed the library using gene specific reverse transcription (Figure 1).

The gene specific library had a significantly greater number (152-fold) of *GABAB1* specific mapped reads than the whole transcriptome library though they have similar total mapped