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Allele specific analysis of the *ADRBK2* gene in lymphoblastoid cells from bipolar disorder patients

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Abstract

G-protein coupled receptor kinase-3 (GRK3), translated from the gene, *ADRBK2* has been implicated as a candidate molecule for bipolar disorder through multiple, converging lines of evidence. In some individuals, the *ADRBK2* gene harbors the A-haplotype, a collection of single nucleotide polymorphisms (SNPs) previously associated with an increased risk for bipolar disorder. Because the A-haplotype encompasses the *ADRBK2* promoter, we hypothesized that it may alter the regulation of gene expression. Using histone H3 acetylation to infer promoter activity in lymphoblastoid cells from patients with bipolar disorder, we examined the A-haplotype within its genomic context and determined that at least four of its SNPs are present in transcriptionally active portions of the promoter. However, using chromatin immunoprecipitation followed by allele-specific PCR in samples heterozygous for the A-haplotype, we found no evidence of altered levels of acetylated histone H3 at the affected allele compared to the common allele. Similarly, using a transcribed SNP to discriminate expressed *ADRBK2* mRNA strands by allele of origin; we found that the A-haplotype did not confer an allelic-expression imbalance.

Our data suggest that while the A-haplotype is situated in active regulatory sequence, the risk-associated SNPs do not appear to affect *ADRBK2* gene regulation at the level of histone H3 acetylation nor do they confer measurable changes in transcription in lymphoblastoid cells. However, tissue specific mechanisms by which the A-haplotype could affect *ADRBK2* in the central nervous system cannot be excluded.

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SN contributed to the experimental work and data analysis.

TB contributed to the study design.

JK contributed to the study design, data analysis and manuscript preparation.

ET contributed to the study design, data analysis and manuscript preparation.

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Keywords

GRK3; Bipolar disorder; Histone; Promoter; Gene expression; Polymorphism

1. Introduction

Bipolar disorder has a well-recognized genetic component, with studies suggesting a monozygotic twin concordance rate approaching 70% (Kendler et al., 1993). Several reports from whole genome studies have suggested multiple disorder-associated SNPs across multiple genes (Sklar et al., 2008; Baum et al., 2008; Ferreira et al., 2008). However, few of these candidate SNPs have been validated for biological function. For this reason, most of the accumulated genetic data for bipolar disorder cannot be prioritized with respect to pathophysiological relevance.

Among the better studied risk-associated genes for bipolar disorder is *ADRBK2*, which encodes for the protein GRK3. Originally detected from linkage studies implicating chromosome 22q13 (Kelsoe et al., 2001; Liang et al., 2002), subsequent sequencing efforts revealed that SNP 28 is over-transmitted among bipolar patients, increasing the relative risk for the disorder by about three-fold (Barrett et al., 2003). Additional reports have expanded upon this finding, suggesting that SNP 28 is in strong linkage disequilibrium with at least five other SNPs, thereby comprising a bipolar disorder-associated haplotype. These SNPs (19, 20, 23, 29, and 32) have been collectively designated as the A-haplotype (Barrett et al., 2007, see Table 1 for dbSNP and alternative SNP designations). Previous work (Zhou et al., 2008) has demonstrated that the rare variant of SNP 28 disrupts Sp1 transcription-factor binding to DNA *in vitro*, and increases *ADRBK2* promoter-driven expression cell transfection models, thereby offering a putative pathophysiological mechanism for bipolar disorder through GRK3.

While strongly suggestive, further characterization of this putative effect with regards to bipolar disorder has been challenging. The A-haplotype extends over several thousand base pairs of the *ADRBK2* locus. To date, only the proximal 550 base pairs of the *ADRBK2* promoter have been examined in assays of transcriptional function, excluding the majority of the SNPs in the A-haplotype. In addition, two large partial gene duplications upstream of *ADRBK2* contain promoter sequences. These duplications are homologous to flanking sequence containing much of the A-haplotype as well as the first exon of *ADRBK2*. It is unclear what role, if any these duplications may play with respect to *ADRBK2* regulation. Finally, efforts to measure effects of the A-haplotype on *ADRBK2* gene expression have been impeded by its rarity (MAF = 0.007), making assays of *ADRBK2* gene expression in brain tissue from bipolar patient autopsies impractical due to the small number of samples available. In such cases sufficient numbers of samples bearing the A-haplotype can only be obtained from lymphoblastoid cells, which have been collected and genotyped for large patient populations.

In the present study we employed two complementary approaches that greatly increase the ability to measure allele-specific consequences of the A-haplotype on the *ADRBK2* gene in tissue from patients with bipolar disorder. These approaches utilize chromatin immunoprecipitation (ChIP) of acetylated histone H3 (ac-H3) at individual *ADRBK2* regulatory alleles and measuring allelic expression imbalances (AEI) among transcribed, synonymous SNPs in the *ADRBK2* transcript.

Recent insights into the role of chromatin structure in gene regulation provide the basis for allele-specific histone modification as a probe for regulatory haplotype function. Specifically, acetylation of histone H3 at lysine residues 9 and 14 is specifically associated with promoters and transcriptional activation across the genome and in multiple tissues, including the brain

(Heintzman et al., 2007; Tsankova et al., 2004; Turner, 2002). Genetic variation can affect both histone modifications and the association of RNA polymerase II at transcriptional start sites (Black et al., 2006; Maynard et al., 2008; Kadota et al., 2007). Therefore, changes to the DNA structure, like those introduced by a SNP, may alter the efficiency of histone-modifying enzymes, perhaps through modification of transcription factor-binding sites, and could thereby influence gene expression. Thus, SNPs associated with the A-haplotype could have an impact upon *ADRBK2* expression by disrupting histone modifications. If so, we predicted that among individuals heterozygous for the A-haplotype, the extent of histone modification at each allele would differ, with the common allele showing more ac-H3 relative to the rare allele. As current evidence suggests that chromatin modifications at promoters are not cell-type specific (Heintzman et al., 2009), these changes may be observable in many different tissues.

AEI has been successfully used to demonstrate allele-specific differences in gene expression a number of candidate gene loci for psychiatric illness including the Phosphatidylinositol 4-kinase type II-beta (*PI4K2B*), serotonin transporter (*SERT*), monoamine oxidase A (*MAOA*) and tryptophan hydroxylase 2 (*TPH2*) (Houlihan et al., 2009; Lim et al., 2006; Pinsonneault et al., 2006; Lim et al., 2007). At the *ADRBK2* locus, assessment of AEI is difficult because the A-haplotype does not include any transcribed SNPs. However, AEI may still be ascertained for the A-haplotype by examining transcribed SNPs from the *trans* allele. If the family pedigree of an A-haplotype proband is informative for the origins of each SNP, AEI in subjects heterozygous for both the A-haplotype and the transcribed SNP can be inferred from quantitative measurements of each mRNA strand at the variant nucleotide.

Using the approaches outlined above, we have characterized bipolar subjects heterozygous for the A-haplotype, analyzing both allele-specific histone H3 acetylation and AEI. While we confirm the A-haplotype affects a transcriptionally active, conserved portion of the *ADRBK2* gene, we find no evidence of difference on promoter utilization or on gene expression as a result of the bipolar disorder-associated haplotype.

2. Materials and methods

2.1 Lymphoblastoid cells

Screening and selection of human subjects with bipolar disorder has been reported previously (Barrett et al., 2003, 2007). Frozen immortalized lymphoblastoid cells obtained from these subjects were reviewed for genotype and availability of DNA samples from family members. From these, 11 cell lines from bipolar disorder patients with the A-haplotype and 6 control cell lines were selected. Because of the rarity of the A-haplotype it was necessary to use multiple subjects from the same family in two cases (2 sets of sibling pairs). Cells were thawed from cryovials and cultured under standard conditions in 10% FBS for 10–14 days to a final density of approximately 1 million cells/ml. At cell harvest, 80% of the sample was fixed in a 4% paraformaldehyde solution at 65° C for 15 min, rinsed in PBS and stored at –80° C until use. The remaining 20% was rinsed in PBS without fixing and stored at –80° C until use for mRNA collection.

2.2 Dissociated fetal brain and Y79 retinoblastoma cells

Human fetal brain tissue was generously provided by Dr Ian Everall, following protocols approved by the Institutional Review Board of the University of California, San Diego. Fetal tissue at 18 weeks gestation was obtained from Advanced Bioscience Resources (ABR, Alameda CA). Whole brain tissue was harvested from the products of conception, mechanically disaggregated, filtered through a 200 µM pore filter and processed to a single cell suspension. Detailed methods for this tissue preparation have been published previously (Trillo-Pazos et al., 2004). Suspension cultures of Y-79 cells were generously provided by Dr Richard Hauger

and prepared as described previously (Hauger et al., 1997). For both cell types, samples were fixed in paraformaldehyde as described above prior to use in ChIP.

2.3 Chromatin immunoprecipitation

Selection of chromatin from lymphoblastoid, brain and Y79 retinoblastoma cells was performed as previously described (Lanier et al., 2007). For each analysis, aliquots of ~10 million fixed cells were suspended in lysis buffer containing 50 mM Tris-HCl, pH 8.1 with 10 mM EDTA, 1% SDS, 1 mM AEBSF and a protease mixture (1X Complete Mini, Roche). Cells were incubated in lysis buffer at 4° C on a rotating platform for 10 minutes. Chromatin was fragmented by sonication, and insoluble cellular debris was removed by centrifugation. Chromatin fragments were diluted in 15 mM Tris-HCl, pH 8.1 with 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, and protease inhibitor mix. An unselected (“input”) sample of 10% of the total homogenate was removed prior to antibody selection and frozen for subsequent DNA recovery.

Ac-H3 ChIP experiments were performed using a commercially available rabbit anti-acetylated histone H3 antibody (Millipore 06–599). For each selection, 25 µg of anti-histone antibody was coupled to 250 µL of sheep anti-rabbit IgG magnetic beads (Dynal M-280). The chromatin was pre-cleared using 25 µL of the magnetic beads with the secondary antibody alone prior to selection in order to reduce non-specific background. The cell sample was incubated overnight with ac-H3 antibody-coupled beads to select the modified chromatin complexes. The selected complexes were washed for five minutes at room temperature with each of the following solutions: 20 mM Tris-HCl, pH 8.1 with 150 mM NaCl, 2 mM EDTA, 0.1 % SDS, and 1% Triton-X-100; 20 mM Tris-HCl, pH 8.1 with 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton-X-100; 10 mM Tris-HCl, pH 8.1 with 1 mM EDTA, 0.25 M LiCl, 1% NP-40, and 1% deoxycholate. Salt and detergent were removed by washing twice with TE, pH 8. DNA was extracted from both the antibody-chromatin complexes and input sample by heating in 0.1 M NaHCO₃ with 200 mM NaCl and 1% SDS at 65° for four hours with constant shaking. The input and selected samples were then digested with proteinase K at 45° C for at least two hours. Yeast tRNA was added to the solution as a carrier to facilitate precipitation. DNA was then extracted with phenol/chloroform, and precipitated with a commercially available co-precipitant (Pellet Paint, Novagen) and ethanol.

2.4 Locus mapping of ADRBK2

ADRBK2 sequence information for a 50 kb section of the 5' promoter region and first exon (Chromosome 22: 24,251,000–24,300,000) was downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov/sites/entrez>). The locations of A-haplotype variants were determined as previously described (Barrett et al., 2003, 2007) and plotted in relation to regions of conserved sequence using the UCSC genome browser, March 2006 assembly (<http://genome.ucsc.edu>). To screen the 50 kb region of interest, 34 PCR primer pairs were designed to sample the 50 kb region of interest at intervals of 1000–3000 bp (Supplementary Table 1). Criteria for PCR primer inclusion were established in control genomic DNA (gDNA): Ct value of 20–25 cycles, a single peak on melting point analysis and recognition of unique sequence using bioinformatic alignment. When ac-H3 enrichment was detected with an initial set of oligonucleotides, additional PCR primer pairs were designed to confirm the result, and increase the density of measurement in a given region. Areas of *a priori* interest (near disorder-associated SNPs, conserved sequence or the transcriptional start site) were also sampled more densely. A total 34 primer pairs met our criteria for use. In a similar manner, 16 PCR primer pairs were designed to sample the two regions of the duplicated promoter sequence upstream of the *ADRBK2* locus (Supplementary Table 1). The sonication process we employ creates chromatin fragments with an average size of about 500 bp, causing the expected ac-H3 ChIP signal to decay over about 1kb in either direction from the site of enrichment. As such, it was

not necessary for the PCR primer pairs to encompass a SNP to give a positive signal as long as the SNP is within 500–1000 bp of the expected PCR product.

2.5 Real-time PCR locus mapping of ac-H3 enrichment

Chromatin fragments recovered from the ChIP-selected and input samples were assayed by real-time PCR in an ABI 7300 thermocycler using SYBR Green fluorimetric detection. ChIP-selected and input samples were run in parallel in a 96-well plate format and signals were measured using the cycle threshold (Ct). Ac-H3 enrichment was assayed by the Ct difference method (Livak and Schmittgen, 2001). The Ct values for each pool of ac-H3 ChIP selected material were normalized to a baseline (1-fold enrichment) determined using the average Ct value for two primer pairs targeting the *ALB* (albumin) gene, which is not transcribed in the cell lines used. Two primer pairs from the *GAPDH* promoter (glyceraldehyde-3-phosphate dehydrogenase) served as positive controls to verify ac-H3 selection (Supplementary Table 1).

2.6 Allele-specific DNA quantification

DNA samples were prepared as described above, generating both input and ChIP-selected materials from the same cell preparations. Four SNPs from the A-haplotype are located in ac-H3 enriched regions of the *ADRBK2* gene. Among these, SNP 23 and 29 were not amenable to TaqMan allele-specific PCR design. For SNP 28 and 32, TaqMan primers (Applied Biosystems) were used to determine Ct values simultaneously for both common and rare alleles of each SNP in both the input and ChIP-selected materials. All samples were run in duplicate and repeated at least twice. PCR discrimination of the common and rare alleles was verified by a difference of at least 5 cycles between Ct values for the target and mismatch probes in DNA from cells that were homozygous for the common allele (i.e. no rare allele present).

Heterozygotes for a particular SNP have a single copy of both the common and rare allele, allowing a within-subject comparison to be used to assay differences in allele-specific histone modification. Preferential H3-acetylation at one allele will be reflected in a biased ChIP recovery efficiency for that allele. Since one cycle difference in Ct corresponds to a two-fold difference in DNA, ChIP recovery efficiency for each allele can be calculated using the formula $R = (2^{\Delta Ct})^{-1} \times 100$ where R is % recovery, and ΔCt is the difference between Ct_{selected} and Ct_{input} . Recovery rates for the common and rare alleles can then be compared to estimate differences in ac-H3. A within subject, two-tailed T-test was used to determine statistical differences between the recovery rates for the common and rare SNP variants.

Standards were generated for SNP 28 and 32 and assayed to estimate the detection sensitivity for changes in ac-H3 ChIP recovery. To prepare these samples, gDNA from heterozygous subjects and from subjects homozygous for the common allele was quantified by PCR using *RNASE P* as a reference. DNA samples from these two sources were then mixed in proportions such that the total DNA concentration was constant, but the resulting fractional abundance of the rare allele was 50% (pure heterozygote DNA), 40%, or 30%. For each SNP, two independent series of standard samples were prepared and five replicate PCR assays were run for each sample. A two-tailed, unpaired T-test was used to test discrimination of each standard sample from heterozygous gDNA containing an equal abundance of the alleles.

2.7 Genotyping

The *ADRBK2* gene contains 41 predicted synonymous and non-synonymous, transcribed SNPs as determined by dbSNP (build 129), viewed using the UCSC Genome Browser. As part of a larger genotyping effort, the 11 subjects containing the A-haplotype were genotyped for each of these transcribed SNPs using the SNPlex Genotyping System with an ABI 3730 48-capillary DNA analyzer according to the manufacturer's directions (Applied Biosystems). Of these 41 SNPs, only rs133839 was present in multiple subjects with informative family pedigrees that

allow for location of the transcribed SNP (*cis* or *trans*) relative to the position of the A-haplotype.

2.8 RNA preparation and cDNA synthesis

RNA extraction from unfixed lymphoblastoid cells was performed using with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Sample quality and RNA concentration were determined using an Agilent 2100 Bioanalyzer (all RNA integrity numbers > 9.6). RNA was treated with RNase-free DNase I following guidelines provided by the manufacturer (Applied Biosystems) prior to cDNA synthesis. cDNA was prepared from 500 ng RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and following the protocol provided by the manufacturer. For each sample, a no reverse transcriptase control was used to verify the absence of gDNA contamination.

2.9 Allelic expression imbalance

TaqMan PCR genotyping primers for SNP rs133839 were purchased from the manufacturer (Applied Biosystems). From each sample heterozygous for rs133839, unselected gDNA was used to normalize the results to correct for minor variations in allelic amplification efficiency, while cDNA samples synthesized from mRNA were measured simultaneously. Both samples from gDNA and cDNA were analyzed in duplicate. Since rs133839 is transcribed into the 3' UTR of the mRNA, it marks each allelic strand of cDNA allowing these to be discriminated in a quantitative manner. The relative abundance of each transcribed allele was calculated by the Ct difference method as described for ChIP recovery assays above. AEI Data are expressed as the ratio of cDNA/gDNA. Significant AEI was defined as a statistical difference between the average cDNA/gDNA ratios for each expressed allele with a $P < 0.05$ using a two-tailed, paired T-test.

AEI Standards were prepared in a manner similar to that described above for ChIP studies. Total *ADRBK2* expression from subjects heterozygous at the rs133839 allele and from subjects homozygous for the common allele was measured by PCR using commercially available primers (Applied Biosystems). Samples of cDNA from these two sources were then mixed in proportions such that the total *ADRBK2* cDNA concentration was constant, but the resulting fractional abundance of the rare allele was 50% (pure heterozygote DNA), 40%, or 30%. Samples were run simultaneously in quadruplicates. A two-tailed, unpaired T-test was used to discriminate each standard sample from the heterozygous sample that contains equal abundance of each allele.

3. Results

3.1 Locus map of *ADRBK2* and ac-H3

There are five bipolar-disorder associated SNPs within the A-haplotype (Barrett et al., 2007). The relationship of these to the transcriptional start site and exon 1 is shown in Figure 1A. In order to understand the relationship of these SNPs to regions of the promoter that are activated during transcription, we designed and validated a large set of PCR primers that tiled a 50kb region of unique sequence along the *ADRBK2* locus at intervals of 500–3000 bp (Methods). We then applied these primers in ac-H3 ChIP assays, and identified a ~10 kb region, centered on the transcriptional start site, that was 30–80 fold enriched in ac-H3 (Figure 1B), comparable to the level of enrichment at two separate *GAPDH* positive control loci (Figure 1C). Similar ac-H3 enrichment was observed in samples with and without the A-haplotype and did not differ by psychiatric diagnosis.

Tiling analysis was also performed for the two upstream regions of partial *ADRBK2* promoter duplication. Primers for these regions were designed as described above, but due to extensive

sequence homology between the *ADRBK2* gene and the two homologous regions, it was not possible to tile the upstream regions with unique PCR primers at the same density as the *ADRBK2* promoter. A total of 11 primer pairs (9 from the proximal homologous region, 2 from the distal homologous region) recognized unique sequence within the two homologous regions. An additional 5 primers recognized unique sequence in the proximal homologous region that did not have sequence homology to *ADRBK2*. None of these probes demonstrated strong evidence of ac-H3 enrichment compared to two *GAPDH* positive control primers (Figure 1D), suggesting that despite considerable sequence similarity to the *ADRBK2* gene, these regions are not transcriptionally active.

The five A-haplotype SNPs were plotted in relation to regions of ac-H3 enrichment as shown in Figure 1. Four of the A-haplotype SNPs (23, 28, 29, and 32) were located in the region of ac-H3 enrichment, suggesting these SNPs lie within transcriptionally active regions of the gene. The fifth, SNP 19 was located outside the region of ac-H3 enrichment by approximately 3 kb.

To better characterize promoter utilization in neuronal tissues, we mapped the same 50 kb portion of the *ADRBK2* locus for ac-H3 enrichment in two additional model systems: Dissociated fetal brain tissue and Y79 retinoblastoma cells, a human neuroectodermal line that has been used extensively to characterize the receptor desensitization activity of GRK3 (Dautzenberg et al., 2002). Both fetal brain and Y79 cells demonstrated a distribution of ac-H3 enrichment across the *ADRBK2* locus that was similar to lymphoblastoid cells (Figure 1). In both neuronal models, the magnitude of ac-H3 enrichment at the *ADRBK2* locus was similar to that observed at the *GAPDH* locus (not shown). Expression of *ADRBK2* mRNA was confirmed in each of the three cells types using an Affymetrix Human Genome U133 Plus 2.0 microarray (data not shown).

An *in silico* analysis using the UCSC genome browser suggested that both SNP 23 and 32 are located in areas of significant inter-species conservation (Figure 1G and Supplementary Figure 1). However, no alterations to consensus transcription factor binding sites were found in these regions as a result of either SNP using the Transcription Element Search System (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>). The regions near SNP 28 and 29 are also conserved across species. The *in silico* analysis of these regions has been published previously (Barrett et al., 2003).

3.2 Allele-specific DNA-histone association

To validate our methodology for allele-specific ChIP, SNP rs220030 was selected as a positive control. SNP rs220030 is located within the *SNRPN* locus, an imprinted gene in which only the paternal allele is expressed, in part as a result of differential H3 acetylation between the maternal and paternal alleles (Gregory et al., 2001). The SNP is common (MAF 30–62%) and four heterozygotes with informative family pedigrees were identified among our A-haplotype bearing subjects. While both the maternal and paternal alleles were readily detected in the input DNA samples, only the paternal allele was efficiently recovered in the ac-H3 ChIP-selected chromatin (Figure 2), confirming that our assay is able to resolve individual polymorphic alleles by their histone acetylation state.

Having established the ability of the assay to recognize allele-specific interactions between ac-H3 and DNA, we applied this technique to two variants within the A-haplotype: SNP 28 and SNP 32. Figure 3 shows the ac-H3 ChIP recovery efficiency for the common and rare alleles of both SNPs. There was no significant difference in ac-H3 recovery between the common and rare alleles for either SNP ($p > 0.30$ in both cases). In contrast, tests using standards prepared with mixtures of A-haplotype heterozygote DNA and common allele homozygous DNA revealed that an allelic imbalance of 40%–60% or greater could be reliably detected for both SNPs ($p < 0.0004$ for SNP 28, $p < 0.0061$ for SNP 32, Supplementary Table 2). Thus, while both

SNP 28 and 32 are in a transcriptionally active region of *ADRBK2*, neither polymorphism leads to changes in this marker of transcriptional activity.

3.3 Allele-specific gene expression

Histone modification *per se* is not the proximal mediator of transcription. Therefore it is plausible that A-haplotype related differences in *ADRBK2* gene expression could occur even without differences in chromatin structure at the promoter. To address differential expression directly, we used AEI for a transcribed *ADRBK2* SNP, rs133839, which is not part of the A-haplotype, but was present in a subset of our subjects. Four of our 11 subjects were heterozygous for rs133839. Among these, family pedigrees were informative for the parental origin of both rs133839 and the A-haplotype for three of them, all of whom were siblings. Pedigree analysis in these cases revealed that SNP rs133839 was on the allele *trans* to the rare variant A-haplotype. The fourth subject's pedigree was not informative with respect to the allelic relationship of the rare A-haplotype to rs133839. However, as the results from this subject did not differ from the other three it was included in the final AEI analysis.

In our A-haplotype samples, amplification efficiency for each rs133839 allele was estimated by PCR in gDNA from four heterozygous subjects, demonstrating that both the common and rare alleles amplified similarly well. To determine the presence of AEI, allele-specific signals were measured in cDNAs generated from the same four subjects, comparing expression of the "tagged" cDNA strand containing rs133839 to the cDNA strand derived from the rare A-haplotype variant. To correct for minor variation in amplification efficiency between the alleles, gDNA Ct values were subtracted from cDNA Ct values for each subject, thereby establishing a reference for equal allelic expression. In all four cases, there was no statistical within-subject difference ($p > 0.30$) in the cDNA:gDNA ratios between the two SNP variants (Figure 4). To test the sensitivity of the AEI assay, we used standard samples with varying amounts of cDNA containing the common and rare rs133839 variants (Methods). In these standard assays, we were able to detect allelic imbalances of 40–60% or greater fractional expression of the favored allele ($p < 0.0003$, Supplementary Table 2). Therefore, we conclude that in lymphoblastoid cells, AEI of 40–60% or greater caused by the A-haplotype at the *ADRBK2* locus can be excluded with good confidence.

4. Discussion

Multiple lines of evidence support *ADRBK2* as a plausible candidate gene for bipolar disorder. GRK3, the protein product of *ADRBK2*, has been reported to change in response to mood modifying drugs like psychostimulants (Niculescu et al., 2000), antipsychotics (Ahmed et al., 2008) and mood stabilizers (Ertley et al., 2007) and *ADRBK2* null mutant mice show attenuated affective responses in the forced swim test, an animal model of depression (Bruchas et al 2007). Finally, in the frontal cortex of patients with bipolar disorder there is a report of diminished GRK3 protein and precursor mRNA (Rao et al., 2009). Taken together, these data strongly suggest that the *ADRBK2* gene may contribute to important neural signaling processes underlying depression and/or mania, the defining components of bipolar disorder.

Given the interest in GRK3 with respect to bipolar disorder, functional characterization of the disorder-associated A-haplotype has been of interest. The A-haplotype also spans conserved, putative regulatory sequence that is conserved across species, suggesting it may be important in regulating the *ADRBK2* gene. In support of this interpretation, we present data that four bipolar disorder-associated SNPs from the A-haplotype are located within a region of functional regulatory sequence, characterized by region-specific ac-H3 enrichment, a mark of transcriptionally active chromatin. The chromatin modification pattern in lymphoblastoid cells was similar to that found in both fetal brain tissue and a well-validated model for GRK3-receptor function, the Y79 retinoblastoma cell. These findings point to overlap in the core

regulatory sequence used by *ADRBK2* across tissues and support the validity of using ac-H3 in lymphoblastoid cells to infer regulatory mechanisms affecting *ADRBK2* in neuronal tissue.

Previous findings have shown that SNP 28 alters a Sp1 transcription factor binding site in the *ADRBK2* promoter and promoter activity in transfection assays (Zhou et al., 2008). Despite this evidence supporting a functional effect of the A-haplotype, we failed to identify any consequence of this SNP or the A-haplotype on either post-translational histone modifications or allele-specific gene expression. Previous studies have also reported discordant results between *in vitro* transfected reporter assays and AEI analysis, suggesting the former approach may inadequately predict gene expression changes caused by allelic variants in their full genomic context (Cirulli and Goldstein, 2007; Wang et al 2008). Furthermore, the Sp1 binding site altered by SNP 28 is only one of 13 Sp1 sites identified by *in silico* assessment of the *ADRBK2* promoter sequence in the region immediately adjacent to SNP 28 (data not shown). Therefore, the effects on chromatin remodeling and transcription resulting from the loss of a single transcription factor binding site may be mitigated by redundant transcription factor activity (both Sp1 and non-Sp1).

Similarly, we cannot exclude the possibility that the A-haplotype has more pronounced and tissue-specific effects on ac-H3 and *ADRBK2* expression in CNS neurons. Indeed, previous authors using approaches similar to ours demonstrated allele and tissue-specific differences in both ac-H3 ChIP and AEI at the *VKORC1* locus, finding differences in the liver, but not in lymphoblastoid cells (Wang et al, 2008); and at the *SERT* locus where AEI was found in the brain but not in lymphoblastoid cells (Lim et al, 2006).

Finally, we also assessed *ADRBK2* gene expression only under baseline conditions. It remains possible that differences in A-haplotype function may only become apparent under conditions that require dynamic regulation of GRK3 (e.g. sustained G-protein coupled receptor activity).

The present study highlights the difficulties faced in interpreting the functional significance of disorder-associated, candidate SNPs. While numerous SNPs have been implicated as putative genetic risk factors for psychiatric illnesses, few of these SNPs affect coding portions of the genome. Most affect presumptive regulatory sequence: promoters, introns, and untranslated regions. Thus, the vast majority of illness-associated SNPs remain uncharacterized with respect to their functional relevance and unconfirmed by independent methodology. Current methods to characterize the function of SNPs in promoter sequence suffer from several key limitations including paucity of appropriate brain tissue, and few assays capable of assessing SNPs in their genomic context.

In the present study, we adapted allele-specific techniques developed in basic genetics and cell biology to assess polymorphic alleles and their putative promoter functions in a candidate gene in samples obtained from a clinical cohort of patients with bipolar disorder. Despite the limitations addressed above, we suggest that allele-specific approaches to regulatory function that preserve genomic and cellular context are an advance over transfection-based techniques and could be widely used in psychiatric genetics. We limited our analysis to a single, well-characterized candidate gene, but propose that the ChIP method in particular could be scaled up to characterize large collections of genetic variants in clinical samples across multiple candidate genes and chromatin modifications, similar to the ChIP-on-chip and ChIP-seq approaches used by others in non-clinical samples (Maynard et al., 2008; McCann et al., 2007). We anticipate that with continued progress in the area of genomics, methods like these will be developed to better characterize and prioritize candidate regulatory SNPs, thereby elucidating their potential pathophysiological roles in mental illness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) 50kb segment of the *ADRBK2* promoter. Box indicates Exon 1. Dots indicate the position of five A-haplotype SNPs from left to right: 19, 23, 28, 29 and 32. (B) Enrichment of ac-H3 across the *ADRBK2* locus in lymphoblastoid cells. Horizontal axis indicates Chr 22 base pair coordinate and vertical axis is fold enrichment compared to *ALB* baseline. Enrichment of ac-H3 did not differ by diagnosis and for purposes of locus tiling, both control and A-haplotype data were averaged together to generate the ac-H3 enrichment profile (N=5) (C) Enrichment of ac-H3 in lymphoblastoid cells at two sites in the *GAPDH* promoter (positive controls) (D) Lack of ac-H3 enrichment at two regions of *ADRBK2* promoter duplication (N=3). Coordinates in A, B and D correspond to chr 22 location, aligned by sequence homology. Vertical lines indicate locations of each SNP (B) or homologous flanking sequence (D) relative to ac-H3 state. (E) Enrichment of ac-H3 across the *ADRBK2* locus in fetal brain tissue. (F) Enrichment of ac-H3 across the *ADRBK2* locus in Y79 retinoblastoma cells. (G) SNP position relative to species conservation at the ac-H3 enriched 10kb section of the *ADRBK2* promoter.

Figure 2.

Allele-specific chromatin recovery was verified using anti-ac-H3 antibodies and allele-specific PCR primers directed at rs220030 from the imprinted gene, *SNRPN*. In the four heterozygotes identified, the paternal allele was more strongly associated with ac-H3, leading to marked imbalances in allelic association with the precipitated chromatin and subsequent recovery efficiency.

Figure 3.

No evidence of allele-specific chromatin recovery at either (A) SNP 28 or (B) SNP 32 in heterozygotes bearing the A-haplotype. Both common and rare alleles were detected by allele-specific PCR in equal amounts after ChIP selection using anti ac-H3 antibodies to precipitate transcriptionally active chromatin. For SNP 28 N=11, for SNP 32 N=8. Three A-haplotype bearing subjects do not carry a rare SNP 32 allele. The differences in averages between alleles were not statistically significant ($p > 0.30$) in either case as measured by a paired two-tailed T-test.

Figure 4.

Allele-specific *ADRBK2* expression was measured in lymphoblastoid cells from four A-haplotype heterozygous subjects using allele-specific PCR probes directed at the transcribed T/C SNP rs133839. The common T allele is *cis* to the A-haplotype. RNA was converted to cDNA and normalized against genomic DNA for each subject. Error bars represent SEM. There was no significant difference in allelic gene expression in any of the subjects. Differences between subjects are due to variability in the content of genomic DNA in the samples, whereas cDNA content between samples was similar.

Table 1

Polymorphic alleles located in proximity to the ~10kb region of ac-H3 enrichment at the *ADRBK2* locus.

NCBI Designation	Barrett et al., 2007 Designation	Other Designations	Chr 22 location (UCS C Build 129)	Sequence
none	A19*		24284271	agtgatgtg[C/T]tgctctgtt
rs2205971	A20		24288952	taatatac[T/C]gtgctgtg
rs41258844	A21	P 7	24289183	gttttaagca[A/C]aaaaaaa
rs5761122	A22	P 8	24289309	ctccttc[A/G]actgccattcc
rs41258274	A23*	P 9	24289311	ctccttga[C/G]tgccattcc
rs41258174	A24	P 1	24289678	ttttaaaca[T/C]aattccagc
rs41262444	A25	P 2	24289702	gtcttcta[A/G]gactgcctgcg
rs41259044	A26	P 3	24289811	acccaggca[T/G]cggggtgg
rs41258004	A27	P 4	24290107	tcttccatcag[A/G]gggctcct
rs41261045	A28*	P 5, G -384A	24290623	ccccgagg[G/A]gagggcgac
none	A29*	P 6	24290890	cgcgcgga[G/-]gggggggct
rs41258164	A30		24291260	gatcggggc[C/G]ggcctctc
rs1988253	A31		24292047	atagttgtc[T/A]ggtaata
rs41258124	A32*	520	24295479	aatggattc[T/C]tagagaca

* Indicates A-haplotype bipolar disorder risk association. Many of the SNPs have been previously discussed using with multiple names as indicated above (Barrett et al., 2001, 2003; Zhou et al., 2008). Chr 22 location corresponds to coordinates from the UCSC genome browser March 2006 assembly.