Published in final edited form as:
Mol Psychiatry. 2009 April ; 14(4): 376–380. doi:10.1038/mp.2008.144.

Singleton Deletions Throughout the Genome Increase Risk of Bipolar Disorder

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Abstract

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An overall burden of rare structural genomic variants has not been reported in Bipolar Disorder (BD), although there have been reports of cases with microduplication and microdeletion. Here, we present a genome wide copy number variant (CNV) survey of 1001 cases and 1034 controls using the Affymetrix SNP 6.0 SNP and CNV platform. Singleton deletions (deletions that appear only once in the dataset) more than 100 kilobases in length are present in 16.2% of BD cases in contrast to 12.3% of controls (permutation p = 0.007). This effect was more pronounced for age at onset of mania ≤ 18 years old. Our results strongly suggest that BD can result from the effects of multiple rare structural variants.

Introduction

Bipolar disorder (BD) is a chronic psychiatric disorder with a worldwide lifetime prevalence of 0.5%–1.5% in the general population.[1] Twin, family, and adoption studies provide evidence of genetic predisposition for BD.[2, 3] Although BD is highly heritable, genome-wide association approaches have yielded limited findings.[4–6]

Rare copy number variants (CNVs, small segmental duplications and deletions), have recently produced encouraging and exciting results for autism and Schizophrenia.[7–12] *De novo* CNVs affecting many loci were identified in 10% or more of sporadic autism patients.[7] Walsh *et al* found that the proportion of individuals with Schizophrenia who carry novel structure variants in Schizophrenia was significantly higher than controls.[10] Rare deletions and duplications or large recurrent deletions increased the risk of Schizophrenia in two other reports.[8, 9] Both family and genetic studies of BD and Schizophrenia suggest shared genetic susceptibility,[13] so it is reasonable to search for association of CNVs with BD.

Systematic genome-wide CNV analysis with BD has not yet been reported. We attempted to identify rare CNVs in people with BD using a SNP- and CNV probe-based platform (Affymetrix SNP 6.0), as part of Genetic Association Information Network (GAIN) project. Our primary hypothesis is that individuals with BD would have a greater genome-wide burden of rare CNVs (either deletions or duplications). Singletons are CNV events that occur once in a data set. In our sample size (1001 patients and 1033 controls) the overall frequency of singletons is roughly comparable to the rare CNVs reported to be increased in disease (up to 6 deletion or duplication events) in the International Schizophrenia Consortium's study (3391 cases and 3181 controls).[9] This led us to focus on singleton events in our analysis (see below).

Methods and materials

Sample recruitment

The study population and subject characteristics are described elsewhere.[14] In brief, this is a genome-wide association case-control study including a European American sample of 1001 Bipolar cases and 1033 controls. The patients were recruited at 11 data collection sites. Patients were interviewed with the Diagnostic Interview for Genetic Studies DIGS,[15] medical records were obtained, and final diagnosis was based on the current APA Diagnostic and Statistical Manual (DSM-IV).[16] Control samples were ascertained separately through a volunteer panel and a web-based psychiatric interview, via a grant awarded Dr. Pablo Gejman and collaborators by the National Institutes of Mental Health (NIMH).

Genotype and CNV detection

Genotyping was carried out by The Broad Institute Center for Genotyping and Analysis (http://www.broad.mit.edu/node/306), as part of a genome-wide association study (GWAS) of BD, with the support of the Genetic Association Information Network (GAIN).[17] The Affymetrix SNP 6.0 array, which was employed, includes 906,600 SNP and 940,000 copy

number probes.[18] Of the CNV probes, 800,000 are evenly spaced along the genome, while the rest are targeted to 3,700 known CNV regions.

The Affymetrix Power Tool (APT-1.10.0, plug-in to Birdsuite 1.5.2) was used to do plate-wise normalization. CNVs were identified using Birdsuite (version 1.5.2; http://www.broad.mit.edu/mpg/birdsuite/analysis.html), which integrates different methods for calling CNVs. Known common CNVs are called with the Canary program, which behaves in a similar way to SNP genotyping, using existing information about known CNVs to train clustering algorithms and assign a prior probability of aberrant copy number to guide interpretation of measurements.[19] The Birdseye program was used to discover rare or *de novo* CNVs, based on a hidden Markov model (HMM);[19] for each CNV a LOD score was generated that describes the likelihood of a CNV relative to no CNV over a given interval including flanking sequences. Global tests of CNV burden in cases versus controls, and association testing of common CNVs, were performed by PLINK (v1.04, http://pngu.mgh.harvard.edu/purcell/plink/).[20]

Quality Control (QC)

QC was performed on SNPs to remove duplicate samples, poorly genotyped and/or contaminated samples, and poorly-performing SNPs, as part of standard quality control measures used for the whole-genome association study of the same sample.[14] The genotype data are available through dbGaP (phs000017.v1.p1).

For 2034 individuals passing QC, we observed 535,553 regions with a copy number other than 2 in autosomes and chromosome X. Chromosome Y was removed from the analysis since the Y chromosome is apparently incorrectly segmented by Birdsuite, version 1.5.2.

In order to generate results with high confidence, we included only CNVs with a physical length greater than 100kb and LOD score ≥ 10. A total of 53,951 segments with a frequency $\geq$10% were identified. We removed outliers based on numbers of frequent CNVs per individual or per chromosome. 51 chromosomes with abnormal CNV estimates were removed (autosomes or chromosome X of females with more than 2.25 copies or less than 1.75 copies and chromosome X of males with more than 1.2 copies or less than 0.8 copy). We also removed individuals with more than 65 common segments, leaving 51,757 common segments.

For rare CNVs, 3 individuals were overrepresented (>10 segments with a frequency <1%). In addition, based on permutation of the frequency of multiple singleton events per individual (from the overall frequency of these events) we excluded 2 individuals with more than 5 singleton deletions and 4 individuals with more than 5 singleton duplications, leaving 1999 individuals (998 cases and 1001 controls, 997 females and 1002 males). These exclusions left 2311 rare CNVs to be analyzed. Among unique, non-overlapping CNVs, there were 310 singleton deletions and 480 singleton duplications.

Plate effects

There were 41 96-well plates in the data set. We tested for plate effects in common CNVs (freq. $\geq$ 10%), by doing a series of GWASs of CNVs by plate. We compared the CNV frequency measured for each plate against that of all other plates in each analysis, using PLINK. Since there were 41 plates, there were 41 tests. The alpha for each GWAS is 0.05, so we would expect approximately 2 positive results by chance.

A similar analysis of plate effect for common CNVs revealed that 560 position markers, from chr8, chr15, chr21, and chr22, showed a plate effect with an EMP2 p-value ≤ 0.05 (familywise p-value across all markers in an analysis), which is many times the expected number and constitutes evidence that there is a plate effect in the common CNV data.

We also did a burden test for plate effects for rare CNVs (frequency <1%, more than 100kb in length and LOD score >10). None of the plates showed a significantly higher number of segments per person (RATE) than all others and none of the plates showed a significantly higher proportion of individuals (PROP) with one or more segments (data not shown).

Quantitative Real Time PCR (qPCR)

We performed quantitative real-time PCR using SYBR-Green dye to measure the copy number of singletons. *HOXA7* was used as an endogenous control. For each region we designed 3 pairs of primers and tested 5 samples (1 with a putative deletion/duplication, the other 4 with putative 2 copies). Since the threshold cycle number (Ct) is a function of the amount of starting template, [21, 22] the relative template amount could be used to identify the copy number. The relative copy number at target regions of the test to the reference is approximately $\{[2^{Ct(\text{target region})}/2^{Ct(HOXA7)}]_{test}/[2^{Ct(\text{target region})}/2^{Ct(HOXA7)}]\}^{-1}_{reference} = 2^{-\Delta\Delta Ct}$.

All the primers designed for qPCR are in Supplementary Table 1. The details of the reactions are in the Supplemental Materials.

Pathway Analysis

We compared genes disrupted in cases versus those disrupted (included in or partially overlapped with the CNVs) in controls using Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, www.ingenuity.com). Genes associated with in the Ingenuity Pathways Knowledge Base were considered for the analysis. Ingenuity uses Fischer's exact test to calculate a p-value determining the probability that each pathway assigned to that data set is due to chance alone.

Results

Singleton deletions increased in BD vs. Controls

The number of singleton deletions per person was significantly enriched in cases vs. controls (0.176 vs. 0.134, p-value=0.010; Table 1). The proportion of cases with at least one singleton deletion was significantly higher than the proportion of controls (16.2% vs. 12.3%, p-value=0.007). The locations and phenotypes of the singleton deletions in the present study are in Supp Table 2. The number of genes disrupted by singleton deletions did not differ between cases and controls (234 genes in cases and 206 genes in controls, p-value = 0.21).

For singleton duplications, no difference was observed between cases and controls. The proportion of cases with at least one singleton duplication was nominally higher than controls (0.324 vs. 0.290, p-value=0.054).

For slightly more frequent rare CNVs (2–6 in dataset of ~2000 individuals), there were no significant differences between cases and controls (data not shown).

Singleton deletions more frequent in earlier onset BD

Considering only patients with onset of mania at age not older than 18 years, the number of segments per person increased from 0.176 to 0.203. The proportion of individuals with at least one singleton deletion increased from 0.162 to 0.189. Considering singleton deletions and duplications together, there was a significant case-control difference in the proportion of individuals with singleton CNVs (p-value = 0.039). The proportion of earlier-onset individuals with at least one singleton deletion is marginally higher than that in later-onset patients (Table 2; p-value = 0.05).

Genes and pathways impacted by singleton deletions

Genes disrupted by CNVs in our cases were significantly overrepresented in pathways categorized by Ingenuity as important for psychological disorders and behaviors like learning (Table 3; p-value = 6.30E-06 and 8.29E-03 respectively). 4 singleton deletion regions overlapped with the novel deletions/duplications that were identified in children but not parents in Walsh *et al*.[10] Two genes affected by CNVs in the present study (*GRM7, LARGE*) were also in the disrupted gene list in the Walsh *et al*. study.[10] No such category was found overrepresented in controls. These overlapping genes could conceivably play a role in risk for both BD and Schizophrenia.

CNV Validation of Singletons by Quantitative Real Time PCR (qPCR)

We randomly selected 19 singleton deletions and 4 gene regions disrupted by singleton deletions/duplications (*GRM7*, *DLG2*, *SOX5*, *LARGE*) to validate by qPCR. *DLG2* and *SOX5* were disrupted by singleton duplications and *GRM7* and *LARGE* were affected by singleton deletions. qPCR confirmed all 23 CNV regions. There were four control individuals for each CNV tested, and no false positives or negatives were observed.

For each region, the copy number was calculated as the mean of $2^{-\Delta\Delta Ct}$ of 3 pairs of primers. The results of RT-PCR for one singleton deletion and one singleton duplication are graphically displayed in Supplemental Figure 1, as an example.

Specific CNVs

Three specific regions (15q13.3, 22q11.2, 1q21.1) have been repeatedly reported to contain rare deletions associated with Schizophrenia.[8, 9, 23] In our own data, because of overlap or proximity to regions with plate effects, we were unable to test these regions for association.

Discussion

The rarest CNVs are interesting because they are presumably enriched in *de novo* events. Under a rare-variant hypothesis of common diseases, where multiple rare variants with high effect sizes would, in aggregate, make a substantial contribution to illness, these would be a prime type of variant to study because they have not yet been subject to selection. There have been several recent studies supporting a very rare-variants hypothesis for Schizophrenia and Autism.[7–12] In the current data, singleton deletions showed a 1.31-fold increase in BD. 16.2% of BD cases possessed at least one singleton deletion, in contrast to 12.3% of controls. Singleton deletions were significantly enriched in BD individuals, unlike slightly more frequent rare CNVs (2–6 occurrences). These data suggest that the burden of very rare (occurring in under 1/2,000 individuals) *de novo* deletions increases risk of BD.

Considering the sample size of the present study, our burden corresponds to the increased singleton and rare deletion (2–6 events) frequencies in Schizophrenia and autism reported in much larger samples in recent publications.[8, 9, 11]

It is interesting that we observe a marginally greater overall burden in BD with earlier age at onset of mania. Among these cases, 18.9% possessed at least one singleton deletion. In contrast, 14.7% of BD cases with older onset carried at least one singleton deletion. We would speculate that early-onset BD might be more severe and more likely to be caused by *de novo* mutations.

Genes disrupted by singleton deletion CNVs in our cases were significantly overrepresented in IPA for psychological disorders and learning behaviors, even though the pathways assembled in IPA may not form a one true biological pathway. One gene (*GRM7*) was implicated in two prior BD GWAS studies.[4, 6] Sixteen genes partially or wholly included in

CNVs in the current study, including CNTNAP2, COMT and *GNB1L*, are included in the IPA psychological disorders pathway. Genomic deletions of the *CNTNAP2* locus in chromosome 7 were found in three patients with Schizophrenia.[24] Catechol-O-methyltransferase regulates dopamine levels in the brain and has long been proposed to be involved in the development of Schizophrenia.[25] *GNB1L* is located in the chr22q11.2 susceptibility region for Schizophrenia, and may be associated BD.[26] Two genes (*GRM7, LARGE*) were also among the list of disrupted genes in the Walsh *et al*. study.[10] There is evidence that BD and Schizophrenia share genetic susceptibility, and the current data suggest they share some rare CNV regions.

We have found suggestive evidence that individuals with BD have a greater burden than controls of singleton deletions across their genomes, which is even greater among patients with earlier onset of mania. Taken together, these singleton findings could account for an appreciable fraction of susceptibility to BD, since the excess frequency of singleton deletions in BD vs. controls is close to 4%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This paper was prepared as part of the Bipolar collaboration of the Genetic Association Information Network (GAIN), and was submitted prior to the end of the publication embargo for non-GAIN scientists. We acknowledge Dr. Dan Nicolae (University of Chicago) for extensive statistical discussion and advice, Mr. Xiaotong Zhang (University of Chicago) for his excellent software support, and Ms. Kay Grennan (University of Chicago) for her technical assistance. This work was supported by grants from 1R01MH081804-01 (NIMH), NARSAD, the Eklund family, and the Geraldi Norton Foundation.

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Table 1

Singletons Burden Test Between BD and Controls

Type	Test	Cases	Controls	P-Value
All	RATE	0.411	0.380	0.156
	PROP	0.324	0.290	0.054
Deletion	RATE	0.176	0.134	0.010
	PROP	0.162	0.123	0.007
Duplication	RATE	0.235	0.246	0.706
	PROP	0.197	0.191	0.378

RATE: Number of segments per person.

PROP: Proportion of sample with one or more segments.

p-value presented: Difference between groups based on 10000 permutations.

Table 2

Singletons Burden Test Between Cases with Mania Onset ≤ 18 yr and Controls, and Between Earlier Onset and Later Onset Cases

Type of CNV	Tested	Cases	Controls	p-value
All	RATE	0.426	0.380	0.145
	PROP	0.343	0.290	0.039
Deletion	RATE	0.203	0.134	0.004
	PROP	0.189	0.123	0.001
Duplication	RATE	0.223	0.246	0.753
	PROP	0.192	0.191	0.498
		Younger onset patients	Older onset patients	
Deletion	RATE	0.203	0.161	0.075
	PROP	0.189	0.147	0.050

The proportion of cases with at least one singleton CNV was nominally higher than controls (0.324 vs 0.290, P-value=0.054

Table 3

Ingenuity Analysis of Disrupted Genes

Category	p-value	Molecules
Psychological Disorders	6.30E-06	*ADH1B, APP, CLDN5, CNTNAP2, COMT, GNB1L, MED15, NRXN1, PI4KA, RCAN2, RTN4R, SNAP29, TXNIP, UFD1L, ZDHHC8, ZNF74*
Behavior (learning)	8.29E-03	*GRM7, PARK2, PTPRD*